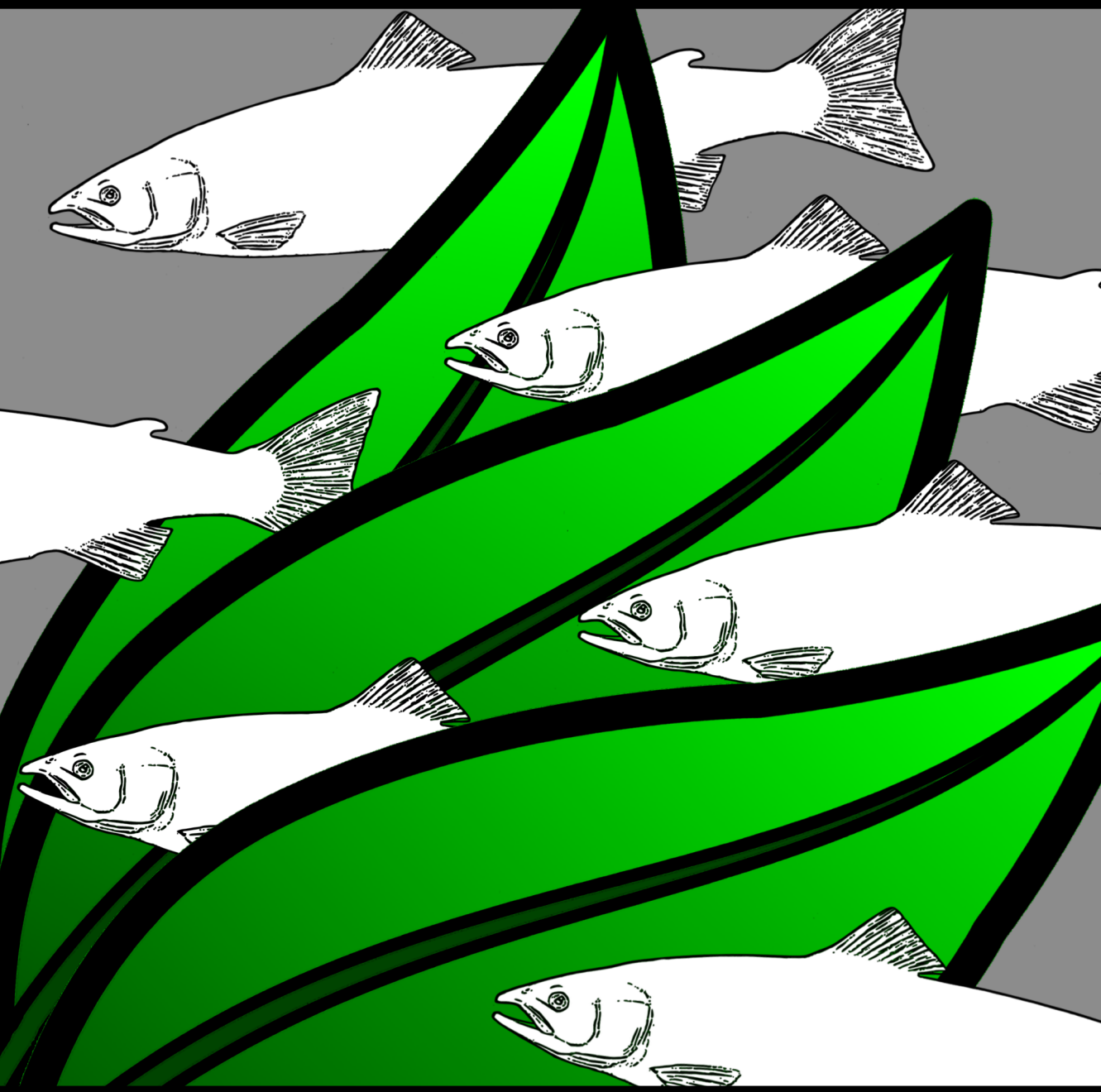
Dietary carbohydrates and denitrification in recirculating aquaculture systems



Andre Meriac

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Dietary carbohydrates and denitrification in recirculating aquaculture systems

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The research was conducted under the auspices of the graduate school of WIAS.

Dietary carbohydrates and denitrification in recirculating aquaculture systems

Andre Meriac

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"If you don't claim your humanity you will become a statistic."
[Fight Club. David Fincher. 20th Century Fox, 1999. DVD.]

Abstract

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Due to overfishing of global fish stocks and increasing fish meal prices, plant ingredients are being increasingly used as an alternative source of protein in fish feeds. However, the inclusion of unpurified plant ingredients will also increase the content of fibers in feeds. Fibers are nearly indigestible and will therefore increase solid waste production in aquaculture. This solid waste can be used to as a carbon source for denitrification to control nitrate levels in recirculating aquaculture systems (RAS), thereby reducing both solid and dissolved waste production. Additionally, fibers can change the recovery characteristics and lower the degradability of fecal waste. Therefore, this study investigates how changes in the dietary carbohydrate composition can affect waste production, system performance and denitrification in RAS. Furthermore, ultrasound treatment (to decrease particle size in fecal waste) and enzymatic conditioning (to increase fiber degradability) were tested as possible means to increase the bioavailability of carbon in fecal waste for denitrification. Comparing a high fiber (HNSP) and low fiber (LNSP) diet in RAS stocked with rainbow trout confirmed that the fibers in the HNSP diet increase fecal waste production. Although the HNSP diet produced more fecal waste than the LNSP diet, both diets produced the same amount of biodegradable fecal carbon. Since feces removal was higher in RAS using the HNSP diet, the load of degradable organic matter on the biofilters was lower with the HNSP diet than with the LNSP diet. Furthermore, fecal waste produced with the HNSP diet contained larger particles than feces of the LNSP diet, which could also improve the recovery of fecal waste with microscreens. Feces produced with the HNSP diet were also less degradable than feces produced with the LNSP diet. By using fecal waste as an internal carbon source for denitrification, solid and dissolved waste emissions from RAS could be reduced by ~50% for the HNSP diet. However, only approximately half of the supplied cellulose and hemicellulose were degraded in the denitrification reactors, whereas lignin was not degraded at all. Thus, the overall degradability of organic carbon in fecal waste was limited by fibers as hemicellulose, cellulose and lignin. Ultrasound and enzymatic conditioning did not sufficiently increase the degradability of fecal waste. Nonetheless, fibers originating from unpurified plant ingredients may also have beneficial effects on RAS performance by increasing fecal recovery. A more selective choice of feed ingredients could be used to increase the recovery and degradability of fecal waste in RAS.

List of abbreviations

ADC	Apparent digestibility coefficient
ADF	Acid detergent fiber
ADL	Acid detergent lignin
BOD	Biochemical oxygen demand (used here as a measure for the biodegradabiliy of organic carbon, determined over 5 or 10 days)
BUN	Branchial and urinary nitrogen losses
CMC	Carboxymethylcellulose
COD	Chemical oxygen demand (indirect measure for organic carbon)
DM	Dry matter
EL	Enzyme loading
FCR	Feed conversion ratio
FW	Fresh weight
HNSP	Experimental diet with high non-starch polysaccharide content
HRT	Hydraulic retention time
HSL	Hydraulic surface load
LNSP	Experimental diet with low non-starch polysaccharide content
MLVSS	Mixed-liquor volatile suspended solids
N	Nitrogen
NDF	Neutral detergent fiber
NFE	Nitrogen-free extract
NFL	Non-fecal loss
NSP	Non-starch polysaccharide
OUR	Oxygen uptake rate
RAS	Recirculating aquaculture systems
sCOD	Dissolved COD
SGR	Specific growth rate
SRT	Sludge retention time
TAN	Total ammonia nitrogen
TS	Total solids
TSS	Total suspended solids
VFA	Volatile fatty acid
VSS	Volatile suspended solids

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1 General introduction

1.1 Background

Global aquaculture production reached a historic milestone in 2011, exceeding global beef production for the first time in modern history (Larsen and Roney, 2013). Although aquaculture can be dated back to as early as 2500 BC, the industrial production of fish is a relatively young discipline when compared to livestock farming (Pillay, 1990). Livestock farming and aquaculture initially relied on the primary production from within the farming environment. Both production systems intensified by using external food sources and controlling the environmental parameters in the production systems. As aquaculture was intensified, the management of waste became crucial to sustain growth and fish performance.

1.2 Waste production in aquaculture

Waste can be defined as feed nutrients, which are not retained in biomass, or otherwise converted into products of value. In fish, feed nutrients can be either used for growth or be excreted as fecal or non-fecal losses (Figure 1.1). When compared to conventional terrestrial animal production, aquaculture by itself is already a very efficient converter of feed nutrients into edible biomass. Production efficiency in aquaculture ranges from 2.5 – 4.5 kg dry feed/ kg of edible biomass, whereas conventional terrestrial animal production requires 3.0– 17.4 kg/kg (FAO/NACA, 2012).
Fecal loss consists of undigested feed nutrients, which can be approximately 10-30% of the feed (Chen et al., 1997). Fecal waste is rich in organic matter and phosphorous, and can cause oxygen depletion and eutrophication when discharged (Iwama, 1991). To minimize the production of solid waste in aquaculture, the general recommendation is to use highly digestible nutrient-dense diets (Cho and Bureau, 1997). Although fish meal is a highly digestible and nutrient-rich protein source, increasing fish meal prices force feed manufacturers to substitute fish meal with unpurified plant ingredients (Bureau and Hua, 2010). However, unpurified plant ingredients often have a lower dry matter digestibility than fish meal (Table 1.1), which is mainly an effect of fibers and other indigestible non-starch polysaccharides (NSPs) in the plant material (Cho and Bureau, 2001; Glencross et al., 2012). Due to the depletion of global fish stocks and increasing world market prices for fish meal, the general prospect is that fish meal will be increasingly replaced

by plant-ingredients (Naylor et al., 2009). Consequently, this will increase solid waste production in fish farming (Bureau and Hua, 2010; Glencross et al., 2012).
Non-fecal losses (NFL) are mostly metabolites of digested feed nutrients, which have been absorbed by the fish but not retained as growth. In fish, the non-fecal loss of nitrogen is excreted as ammonia and urea. Due to its toxicity for fish and eutrophication potential, ammonia is especially of interest for the aquaculturist (Iwama, 1991; Tomasso, 1994). Non-fecal nitrogen losses are the result of protein catabolism, and can vary between 30-65% of feed N (Schneider et al., 2005). This loss can be reduced by providing the fish with a diet, which has an optimal amino acid profile and digestible protein/digestible energy ratio (Bureau and Hua, 2010). However, nitrogen retention also depends on fish size and will be the highest in fast-growing juveniles (Azevedo et al., 2004).

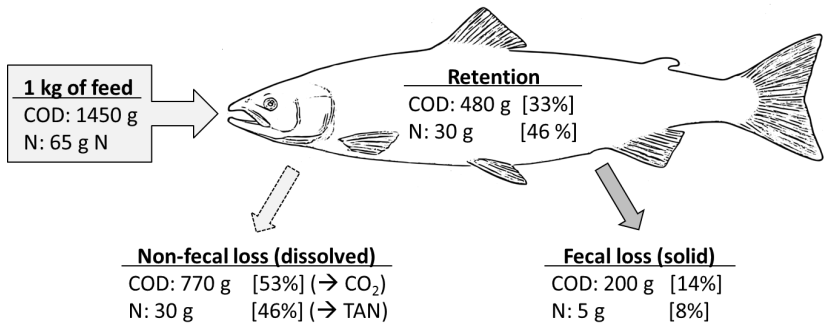

Figure 1.1: Exemplified COD and N mass balance for fish. Estimated from Meriac et al. (2014), based on rainbow trout. Approximately 200 g of fecal waste are produced per kg of feed (COD/DM ~1, Nijhof, 1994). COD: chemical oxygen demand, used as a measure for organic carbon; TAN: total ammonia nitrogen.

Table 1.1: Apparent digestibility of selected feed ingredients in rainbow trout (Cho and Bureau, 1997, citing Cho and Kaushik, 1990). The digestibility of unprocessed plant material can increase during the extrusion of the feed (Cheng and Hardy, 2003).

	Apparent digestibility [%]		
Ingredient	**Dry matter**	**Crude protein**	**Energy**
Fish protein concentrate	90	95	94
Blood meal (spray-dried)	91	99	89
Fish meal (herring)	85	92	91
Soy protein concentrate	77	97	84
Soybean meal	74	96	75
Alfalfa meal	39	87	43
Wheat middlings	35	92	46
Rapeseed meal	35	77	45

1.3 Waste management and water re-use in aquaculture

The simplest way to manage the accumulation of solid and dissolved waste in the culture systems is the renewal/exchange of water. However, this is only possible as long as there is sufficient water available, as for example in ponds, raceways or sea cages. Because of the potential environmental impact of untreated waste on the environment, this practice remains questionable and is closely scrutinized in areas where aquaculture meets the public domain (Bureau and Hua, 2010; Iwama, 1991). Stringent environmental laws and levies on water use and waste discharge promoted the development of re-use systems, in which water is (at least partially) re-used after purification (Klinger and Naylor, 2012; Piedrahita, 2003). Essentially, re-use systems need to control the accumulation of waste within system while providing the fish with feed and oxygen to ensure optimal growth. Re-use systems as recirculating aquaculture systems (RAS) became popular in fish culture due to the following advantages:

- Efficient use of water, reducing water demand by 90-99% when compared with flow-through systems (Table 1.2)(Timmons and Ebeling, 2007; Verdegem et al., 2006).
- Control over environmental parameters in closed systems, allowing for optimal growth conditions independent of season (Timmons and Ebeling, 2007).
- Supreme standards for biosecurity in closed systems, reducing the chance of introducing pathogens or releasing escapees into an ecosystem (Sharrer and Summerfelt, 2007; Zohar et al., 2005).

Although the contribution of RAS to the total aquaculture production is still small compared to open systems, RAS are gaining significance for the production of high-value species and fingerlings for stocking (Martins et al., 2010; Dalsgaard et al., 2013).

Table 1.2: Water requirement and production intensity in different aquaculture systems (adapted from Timmons and Ebeling, 2007). Production intensity and water requirement is based on kilograms of biomass production.

System	Species	Production intensity [kg/ha/y]	Water required [L/kg]
RAS	Nile tilapia (*O. niloticus*)	1,340,000	500
Pond	Nile tilapia (*O. niloticus*)	17,400	21,000
Pond	Channel catfish(*I. punctatus*)	3,000	3,000-5,000
Raceway	Rainbow trout (*O. mykiss*)	150,000	210,000
Pond	Panaeid shrimp	4,2000-11,000	11,000 – 21,340

1.4 Design and operation of RAS

The basic RAS design is relatively simple and consists essentially of three key components, which are [i] the rearing units [ii], a solids separation unit and [iii] a biofilter (Figure 1.2) (Bovendeur et al., 1987; Liao and Mayo, 1974). More sophisticated systems use oxygen injection at the tank inlets to increase the carrying capacity of the systems, and/or may use further treatment steps to improve water quality with UV treatment, ozonation and/or skimming (Timmons and Ebeling, 2007).

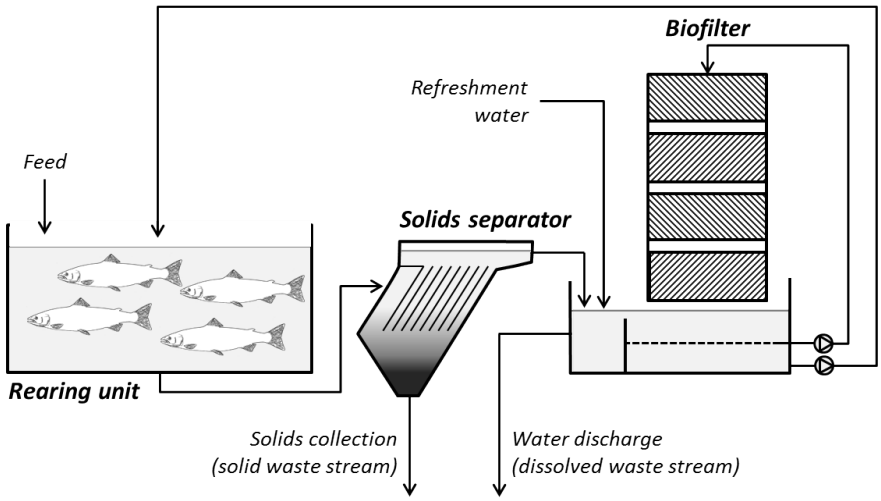

Figure 1.2: Basic RAS design, using a lamella separator for solids removal and a trickling filter for nitrification.

The first and most important step of waste management in RAS is the removal of solid waste. Although solid waste mainly consists of fecal waste, it can also contain feed spill, detached biofilm or fish tissue (Timmons and Ebeling, 2007). Failing to adequately remove solid waste can impair fish welfare and hamper biofilter performance (Bovendeur et al., 1990; Chapman et al., 1987). Solid waste separation is usually done by either gravity (sedimentation), size exclusion (microfiltration), or even a combination of both (Summerfelt and Penne, 2005; Twarowska et al., 1997). Sedimentation can usually only remove particles larger than 100 µm effectively, smaller particles can be removed by microscreen filtration (e.g. drum filters)(Timmons and Losordo, 1994). Consequently, the effective removal of solid waste from the systems is sensitive towards particle size, stability and density (Brinker et al., 2005a; Couturier et al., 2009; Wong and Piedrahita, 2000). These characteristics are heavily dependent on diet composition, and changing diet composition can heavily affect the solid waste removal efficiency (Amirkolaie et al., 2006, 2005; Ogunkoya et al., 2006).

The second step of waste management in RAS is the conversion of excreted ammonia to less-toxic nitrate. Different types of fixed-film biofilters are used in RAS, of which all have the purpose to provide oxygen and surface area for the nitrifying biofilm (Timmons and Losordo, 1994). However, the performance of the biofilter is sensitive to the load of organic matter (Figure 3), which can be expressed as either chemical oxygen demand (COD) or biochemical oxygen demand (BOD) (Bovendeur et al., 1990; Ling and Chen, 2005; Zhu and Chen, 2001). COD from unrecovered fecal waste can favor the growth of heterotrophic bacteria in the biofilter, which will out-compete the autotrophs and eventually hamper nitrification efficiency (Michaud et al., 2006). Nitrate, unlike ammonia, has a low toxicity to aquatic organisms, and critical concentrations for some fish species can be even above 400 mg/L NO_3^- (Timmons and Ebeling, 2007; Tomasso, 1994). However, nitrate accumulation needs to be counteracted to avoid toxic concentrations in the system, which is usually done in RAS by exchanging water. Accordingly, the production of nitrate eventually determines the minimal water exchange rate of a RAS, which can be as low as 100L/kg feed (Martins et al., 2010, citing Eding and Kamstra, 2002).

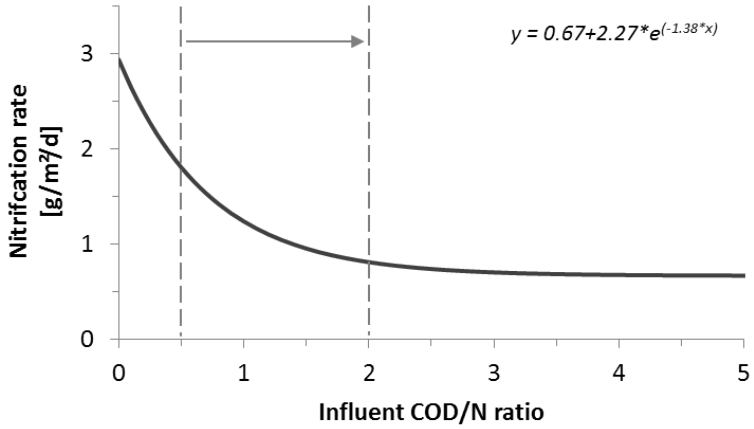

Figure 1.3: Relationship between the nitrification rate and the influent COD/N ratio in biofilters (Chen et al., 2006). Increasing the influent COD/N ratio from 0.5 to 2 reduces the nitrification rate by 55%. To maintain equal nitrification capacity, the biofilter surface would need to be doubled.

1.5 Waste treatment in RAS

In locations where waste discharge is regulated and can limit fish production by either discharge quotas or fees, waste treatment becomes necessary. In contrast to open systems, the low discharge volumes, together with a concentrated solid waste stream, now offer the possibility to treat the waste leaving the recirculation systems (Verdegem, 2013). Waste emission from aquaculture systems can be minimized by either [i] reducing waste production through feed formulation, [ii] converting the residual nutrients into products of value (e.g. shellfish, bioflocs, algae), or [iii] "destroying" the residual nutrients (Table 1.3).
Although the conversion of nutrients into valuable products is preferable, this is often not possible in intensive systems due to space limitation, additional management or low market value of the secondary products. The use of solid waste as a fertilizer in agriculture usually requires additional thickening and storage of sludge, which also results in additional management and costs (Bergheim et al., 1998; Cripps and Bergheim, 2000; van Rijn, 2013). Furthermore, this is also dependent on location and not always possible for marine farms due to the high salt content of the solid waste (Chen et al., 1997; Mirzoyan et al., 2010). Therefore, the on-site treatment of waste is often an attractive way for fish farmers to reduce waste emissions (van Rijn, 2013). The use of denitrification in internal carbon sources was proposed as an elegant way to reduce solid and dissolved waste emissions from RAS, while offering the possibility to control nitrate levels in the culture systems (Gelfand et al., 2003; Kaiser and Schmitz, 1988; Schuster and Stelz, 1998; Shnel et al., 2002).

1.6 Denitrification in RAS

Denitrification is an anoxic microbial process, using organic carbon to convert nitrate into inert nitrogen gas (Equation 1.1)(Tchobanoglous et al., 2004). Although denitrification can be applied as an end-of-the-pipe treatment, it is mostly used within the recirculation loop to control nitrate levels (Timmons and Ebeling, 2007). Denitrification requires a carbon-to nitrogen ratio of 3- 6 g COD/g NO_3-N (van Rijn et al., 2006). Within RAS, denitrification reactors are usually operated with external carbon sources like methanol or acetate to provide sufficient carbon for adequate nitrate removal (Balderston and Sieburth, 1976; Sauthier et al., 1998).

$$5\,CH_3OH + 6\,NO_3^- \longrightarrow 3\,N_2 + 5\,CO_2 + 7\,H_2O + 6\,OH^- \quad (1.1)$$

[Denitrification, using methanol as a model substrate (Tchobanoglous et al., 2004).]

Several authors have successfully used fecal waste as an internal carbon source for denitrification (Gelfand et al., 2003; Kaiser and Schmitz, 1988; Schuster and Stelz, 1998;

Table 1.3: Common waste reduction and management strategies in aquaculture.

Treatment	Mode of action	Advantages	Disadvantages	Literature
Prevention				
Feed formulation	Diet optimization, increasing nutrient retention in fish	Increased productivity, less need for treatment	High feed prices	Bureau and Hua, 2010; Cho and Bureau, 1997
Conversion into valuable products				
Biofloc technology	Conversion of waste into bioflocs and used as fish feed	Waste conversion into fish biomass	Species- & system dependent, low nutritional value of flocs	Azim and Little, 2008; reviewed by Crab et al., 2012
Integrated-multi-trophic systems	Co-culture of e.g. shrimp, shellfish or algae	Nutrient conversion into valuable products	Footprint, requires good management & market for products	Corey et al., 2014; Metaxa et al., 2006; Shpigel et al., 1993
Anaerobic digestion	Anaerobic digestion of solids for biogas production	Biogas production	Often not feasible on-site, low methane potential	reviewed by Mirzoyan et al., 2010
Land application	Use of solid waste as fertilizer in agriculture	Nutrient recycling	Location-depen dent, requires thickening, limited applicability for saline sludge	Naylor et al., 1999; Willett and Jakobsen, 1986
"Destruction"				
Constructed wetlands	Using a biological system to break down nutrients	Low maintenance	Footprint, efficiency can vary with season	Schulz et al., 2003; Summerfelt et al., 1999
Sludge digestion basins	Bacterial breakdown of COD	Low maintenance	Footprint, odor, management of residual solids	reviewed by Chen et al., 1997
Geotextile filtration	Solids storage & composting in net bags	Low maintenance, dewatering, mineralization	Use of coagulants/ flocculants, odor, management of residual solids	Guerdat et al., 2013; Sharrer et al., 2009
Denitrification on internal carbon sources	Using solid COD to convert nitrate to N_2	Footprint, nitrate control, alkalinity production	Management of reactor & residual solids	Gelfand et al., 2003 Kaiser and Schmitz, 1988 Martins et al., 2009

Shnel et al., 2002). Using internal carbon sources does not only reduce the expenses for external carbon sources, but also reduces solid waste production in RAS (SustainAqua, 2009). Furthermore, denitrification produces alkalinity, which can be used in nitrification (Tal et al., 2009; van Rijn et al., 2006). By controlling nitrate levels with denitrification on internal carbon sources, the water demand of RAS can be reduced to 30 L/kg feed (Martins et al., 2009). The main limitations of using internal carbon sources in denitrification is [1] the quantity of fecal waste (Kaiser and Schmitz, 1988; Klas et al., 2006a) and [2] the quality of the fecal waste (Klas et al., 2006a). The quantity of fecal waste, which can be used for denitrification in RAS, is determined by the digestibility of feed by the fish and the efficiency of the subsequent solid waste recovery. The quality of the solid waste is determined by the bioavailability of carbon, which determines how much of the total carbon can be utilized by the bacteria. Carbon bioavailability can be limited by the low degradability of fibers like hemicellulose, cellulose and lignin or other NSPs originating from plant material (Hendriks and Zeeman, 2009; Noike et al., 1985). If carbon will become limiting, conditioning methods like ultrasound or enzyme treatment could be applied to increase the amount of bioavailable carbon for denitrification (McDermott et al., 2001; Wen et al., 2004). Consequently, the total efficacy of denitrification on internal carbon sources depends on feed digestibility, fecal waste recovery and the bioavailability of carbon in fecal waste (Figure 1.4). Changing feed ingredients can have a severe impact on those turning points, and therefore change the nutrient flows along this feed-fish-waste axis.

1.7 Problem definition and objectives

Feeding fish will result in the production of a solid and dissolved waste stream, which has to be managed within a husbandry system and should be treated before it is discharged into the environment. Apart from general environmental concerns, the costs of waste treatment, management and discharge can impose a significant financial burden on fish farmers. Denitrification on internal carbon sources was identified as a suitable means to reduce waste production in RAS. However, the trend to substitute fish meal in fish feeds with un-purified plant ingredients will induce a shift in the dietary carbohydrate composition, increasing the amount of indigestible fibers in feeds. Changing feed composition can have major repercussions on the feed-fish-waste axis, ultimately affecting the performance and overall waste emissions of RAS (Figure 1.4). Although the effect of fibers on digestibility and fecal waste recovery has been investigated independently, there is a lack of studies which address this issue in a systemic context for RAS. Furthermore, the potential of increasing carbon bioavailability for denitrification by solid waste conditioning remains yet to be explored in RAS. Therefore, this thesis should provide insights into how dietary fibers affect digestibility, solid waste recovery and carbon bioavailability in RAS, and propose solutions for the future challenge of an increased substitution of fish meal with plant-based ingredients.

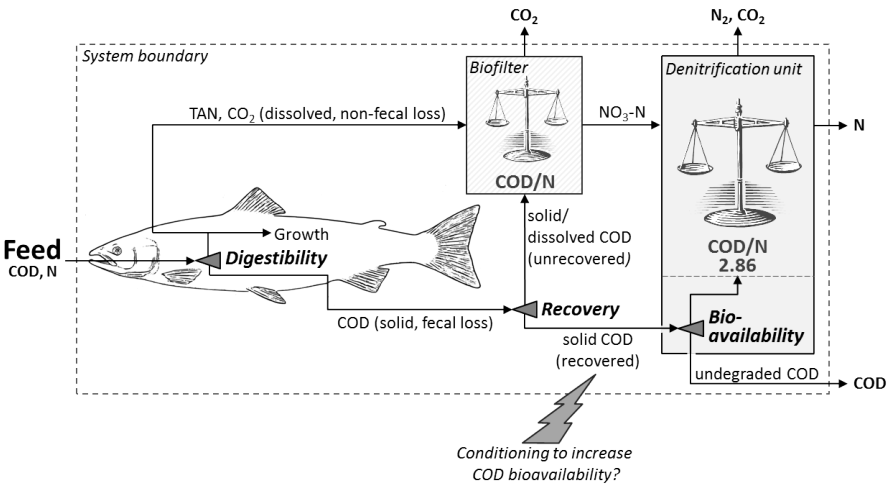

Figure 1.4: COD and N dynamics along feed-fish-waste axis. Digestibility, recovery and bioavailability are the key points, in which diet composition can influence system performance and the efficacy of denitrification on internal carbon sources. COD and N, which was neither retained by the fish nor converted to CO_2 or N_2, leaves the system as waste. To reduce 1 g of nitrate-N in denitrification, 2.86 g of bioavailable COD need to be oxidized (Tchobanoglous et al., 2004). Bottlenecks in COD bioavailability could be resolved by applying conditioning methods.

1.8 Content of this thesis

This thesis aims to answer the question how an increased fiber content in fish feeds will affect waste production, system performance and waste treatment potential in RAS. Therefore, we investigated in the following research chapters whether an increasing dietary fiber content:

- Increases the production of solid waste in RAS (Chapter 2, 4);
- Increases or decreases the recovery of fecal waste in sedimentation (Chapter 2) or microscreen filtration (Chapter 3);
- Increases the organic load on the biofilters (Chapter 2);
- Increases or decreases the total amount of bioavailable carbon for denitrification (Chapter 4).

Furthermore, we investigated whether the bioavailability of carbon in fecal waste can be increased by:

- Ultrasound treatment, to reduce particle size in fecal waste (Chapter 3), or;
- Enzymatic conditioning, to convert fibers into readily available carbon sources (Chapter 5).

The final chapter contains a brief summary and a discussion of the main outcomes of this thesis. The discussion should provide insights into how fibers in feed can affect the current waste treatment concept in RAS. Furthermore, this chapter also contains a number of recommendations which can help to meet the future challenges of fish meal substitution with unpurified plant ingredients.

2 Dietary carbohydrate composition can change waste production and biofilter load in recirculating aquaculture systems

Published as

Meriac, A., Eding, E. H., Schrama, J. W., Kamstra, A., & Verreth, J. A. J. (2014). Dietary carbohydrate composition can change waste production and biofilter load in recirculating aquaculture systems. Aquaculture, 420-421, pp. 254–261.

Abstract

This study investigated the effect of dietary carbohydrate composition on the production, recovery and degradability of fecal waste from rainbow trout *(Oncorhynchus mykiss)* in recirculating aquaculture systems (RAS). Dietary carbohydrate composition was altered by substituting starch with non-starch polysaccharides (NSPs) while keeping the diets isonitrogenous and isoenergetic. We tested a high starch, low NSP (LNSP) and a low starch, high NSP (HNSP) diet in six identical, small-scale RAS (V= 460 L). Each diet was tested in three independent systems over a period of six weeks. Shifting dietary carbohydrates from starch to NSPs resulted in a 50% increase in the production of chemical oxygen demand (COD) based on digestibility. Fecal waste recovery showed a 40% increase in HNSP treatments when compared with LNSP. Consequently, the COD output from HNSP systems doubled from 91 g to 194 g of COD per kg feed when compared with LNSP. Although COD production was higher in HNSP systems, the COD load on the biofilters was significantly lower when compared with LNSP systems. COD-to-nitrogen (COD/N) ratios in the biofilter load were 1.7 $\pm$ 0.2 and 2.2 $\pm$ 0.2 g COD/g N for HNSP and LNSP, respectively. Shifting the dietary carbohydrate composition from starch to NSPs decreased the biodegradability of fecal COD from 66.3% to 43.7% ($p<$ 0.001). Fiber analyses revealed that approximately 40% of the COD in HNSP feces came from cellulose and hemicellulose. The increased COD production of HNSP diets could be exploited by using fecal COD as an internal carbon source in denitrification. Full denitrification would be theoretically possible with a measured COD/N ratio of 7.2 in the waste stream of HNSP systems. However, it is not clear if the low COD bioavailability of HNSP feces could be a limiting factor. This study shows that COD/N ratios in the biofilter load and system output can be manipulated by changing dietary carbohydrate composition. Although an increased dietary NSP content increased COD production, it also increased COD recovery, decreased COD load on the biofilters and generated sufficient carbon for denitrification on internal sources.

2.1 Introduction

Over the past decades, many studies have shown how plant-based ingredients can be used to substitute fish meal in fish feeds without significant losses in fish performance (El-Saidy and Gaber, 2003; Fournier et al., 2004; Gomes et al., 1995; Kaushik et al., 1995; Lund et al., 2011). The process of feed formulation focuses almost entirely on protein efficiency and the allocation of energy, using the most economical combination of ingredients (Pillay, 1990). Depending on the ingredients used, the carbohydrate fraction in the feeds can contain different levels of non-starch polysaccharides (NSPs), which directly affect dry matter (DM) digestibility and fecal stability (Amirkolaie et al., 2005; Glencross, 2009; Hilton et al., 1983). Increasing the indigestible NSP content of a diet will thus increase the net production of chemical oxygen demand (COD) per unit of nitrogen (N) produced (Farhangi and Carter, 2007; Glencross et al., 2012). The basic treatment of water in recirculating aquaculture systems (RAS) requires the removal of solid COD and the complete conversion of ammonia-nitrogen into nitrate (Timmons and Ebeling, 2007). An incomplete removal of solid waste can result in an excessive load of COD on the biofilter, hampering the nitrification process (Zhu and Chen, 2001). The effective load of biochemical oxygen demand (BOD) is determined by the degradability of the supplied COD. Lignocellulosic material originating from unpurified plant ingredients can significantly decrease COD degradability. A decreased COD degradability will restrict the amount of readily degradable carbon for possible waste treatment processes like denitrification. Several authors have proven that the solid organic waste generated in RAS can be used as a carbon source for denitrification (Gelfand et al., 2003; Kaiser and Schmitz, 1988; Schuster and Stelz, 1998; Shnel et al., 2002). Using denitrification on internal carbon sources does not only reduce COD and N output from RAS, but it even allows to lower the water exchange to 30 L/kg feed in RAS (Martins et al., 2009). To the best knowledge of the authors, there were no papers in literature which specifically discuss the effect of an increased NSP content in feeds on [1] COD production, composition and recovery, [2] COD and N load of the biofilters and system performance, and [3] COD/N ratios and COD bioavailability for denitrification. Therefore, we investigated the effect of a low starch, high NSP diet (HNSP) and a high starch, low NSP diet (LNSP) on COD and N mass balances on fish level and system level. The goal of this experiment was to determine the COD and N load on the biofilters (system load), in-situ losses (system losses) and the possible denitrification potential in the waste stream leaving the experimental RAS (system output).

2.2 Material and Methods

2.2.1 Experimental design

We tested the effect of an NSP-rich diet on COD and N production, COD/N ratios and solid COD degradability in a total of six independent RAS stocked with rainbow trout *(Oncorhynchus mykiss*, Walbaum 1792). The experimental period was six weeks, two experimental diets were used as treatments and each treatment was replicated three times. The two experimental diets were a low starch, high NSP diet (HNSP) and a high starch, low NSP diet (LNSP). The experiment was approved by the Ethics Commission for Animal Experiments of Wageningen University and is filed under the reference number 2011085.c.

2.2.2 Replicated recirculating aquaculture systems

System configuration: Each replicated RAS (Figure 2.1) was composed of a circular fish tank (V=300 L), a settling cone (V=75 L, hydraulic surface load (HSL) 150 $m^3/m^2/d$; Fleuren & Nooien, Nederweert The Netherlands), a sump (V=75 L) equipped with a UV unit (UV-C 36 W, Phillips, Eindhoven, The Netherlands) and two trickling filters of equal size and surface area (A=15.8 m^2 for each filter, cross-flow medium, 242 m^2/m^3 specific surface area, Fleuren & Nooien, Nederweert, The Netherlands). One biofilter was operated in bypass across the sump with a flow of 6-7 L/min (HSL: ~85 $m^3/m^2/d$), the other filter was located above the fish tank and loaded with the main flow of 20 L/min (HSL: 264 $m^3/m^2/d$, see Figure 2.1). The total volume of each RAS was 460 L. Each fish tank was equipped with a double stand pipe for solids removal from the bottom of the tank. The fish tank effluent passed the settling cone with minimal head loss. The feces settled into a water-cooled glass bottle (T= 4 °C), which was attached to the bottom of the cone. The water supply of the systems was connected to the sump and the exchange water was discharged using a tap at the bottom of the sump. Each of the six RAS was randomly assigned to either the HNSP or LNSP diet.
System acclimatization: To develop the necessary nitrification capacity in the experimental systems, pre-cultivated cross-flow biofilter media was supplied with NH_4Cl for 10 days. Afterwards, each RAS was stocked for 12 days with 24-25 (non-experimental) trout to adapt the biofilters to the feed load of the experiment. The conductivity of all systems was gradually increased with artificial sea water (Instant Ocean, Aquarium Systems; Sarrebourg, France) to ~2.1-2.2 mS/cm.
System operation: The systems were operated at a temperature of 15-16 °C, sodium bicarbonate was added when necessary to keep the pH between 7 and 8 and the photoperiod was set to 12:12 h light/dark. Water losses due to evaporation were compensated in the mornings before water exchange and feeding. Water was exchanged daily before the morning feeding at a rate of 450 L/kg feed, the exchange volume was based on the feed

load of the previous day. Conductivity was maintained between 1 and 2 mS/cm by the addition of artificial sea water during the experiment.

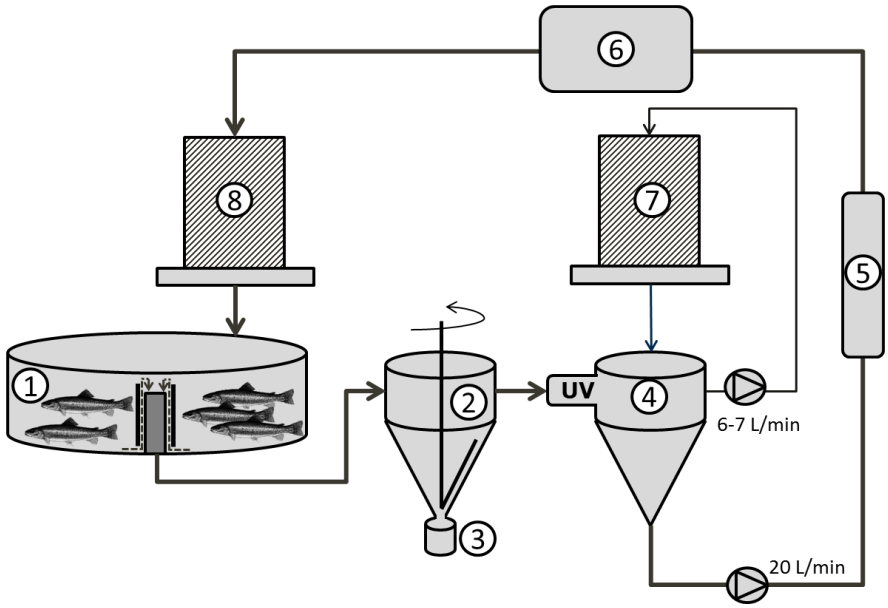

Figure 2.1: RAS layout; [1] fish tank with double stand pipe (V=300L, A=0.72 m^2), [2] settling tank /w stirrer to clean tank walls every 30 min (V=75L, HSL: 150 $m^3/m^2/d$); [3] Collection bottle connected to settling tank and cooled to 4 °C (V=250 ml); [4] Sump (V=75L) and UV (UV-C 36 W, Phillips, Eindhoven, The Netherlands), water exchange and sampling point; [5] flow meter; [6] cooler-heater (TC20, Teco, Ravenna, Italy), [7] & [8] trickling filter /w cross flow medium (V=0.059 m^3, A= 15.8 m^2 each)

2.2.3 Fish

Rainbow trout (*Oncorhynchus mykiss, Walbaum 1792*) were obtained from a trout farm in Germany (Mohnen GmbH, Stolberg, Germany) and housed at the experimental facilities of "De Haar Vissen" (Wageningen, The Netherlands). The fish arrived at an age of ~6.5 months and a bodyweight of ~75 g. Upon arrival, fish were acclimated in circular tanks on flow-through supplied with well water of 13-15 °C. Two weeks prior to the start of the experiment, the fish were divided into an HNSP and a LNSP group and housed in separate tanks. Each group was fed with their respective diet at the same levels as during the experiment for acclimatization, and feeding was ceased one day prior to the start of the experiment. Experimental fish from either the HNSP or LNSP adapted group were stocked in recirculation systems assigned to their respective diet. The fish had an individual average weight of ~100 g, resulting in an initial stocking density of ~9.5 kg/m^3

for all systems.

2.2.4 Diets and Feeding

The fish were fed two experimental diets (Table 2.1), which were formulated by our work group and produced by Research Diet Services (Wijk bij Duurstede, The Netherlands). The diets were fed as extruded floating pellets of 3 mm, and contained acid insoluble ash (AIA) as a marker for indirect digestibility estimation (Atkinson et al., 1984; Vandenberg and De La Noue, 2001). Nutrient digestibility was estimated based on the solid waste collected in the settling cones of each RAS, assuming that the contribution of solids from RAS is negligible when compared to the amount of fecal waste collected (Farhangi and Carter, 2007; Saravanan et al., 2012; Thiessen et al., 2003). The feeds were designed to be isonitrogenous and isoenergetic based on digestibility. The main difference between the feeds was the allocation of digestible energy. The bulk of the digestible energy in the LNSP diet was allocated in starch and in highly digestible fat for the HNSP diet. Fish were fed at an average of 1.5% of body weight per day, the daily ration was based on an assumed FCR of 1. Prior to feeding, feed fines were removed by sieving. The daily feed ration was divided equally between the morning feeding (10:00) and the afternoon feeding (16:00).

Table 2.1: Feed ingredients as ordered at the manufacturer.

Parameter	Unit	HNSP	LNSP
Fish meal	[g/kg]	250	410
Fish oil	[g/kg]	82	22
Soy bean meal	[g/kg]	150	0
Wheat flour	[g/kg]	175	530
Sunflower seed meal	[g/kg]	150	0
Rape seed meal	[g/kg]	150	0
Monocalciumphosphate	[g/kg]	5	0
L-Lysine HCL	[g/kg]	4	4
DL-Methionine	[g/kg]	4	4
Diamol	[g/kg]	20	20
Premix[a]	[g/kg]	10	10

[a] Premix, includes vitamins, minerals and trace elements

2.2.5 Sampling

Sieved feed samples of 100 g of the HNSP and LNSP diet were collected once a week, pooled and stored in an airtight container at 4 °C for later proximate analysis. A random

sample of ten fish to determine initial body composition was taken from each the HNSP and LNSP adapted groups immediately prior to the stocking of the experimental RAS. The fish were euthanized with 2-phenoxy-1-ethanol (1 ml/L) and stored at -20 °C in sealed bags until analysis. At the end of the experiment, all fish were euthanized with 2-phenoxy-1-ethanol (1 ml/L) and weighed individually. Ten fish were randomly sampled from each replicate and stored at -20 °C for proximate analysis of final body composition. The settled feces were collected twice a day before feeding. The 250 ml collection bottles were disconnected from the settling cone and the supernatant was decanted back into the systems. The remaining solids were collected in aluminum trays and stored at -20 °C for later analysis. Only the fecal waste samples collected during the last two weeks of the experiment were used for the determination of solids composition, digestibility and recovery. Water samples were taken at the start of the experiment, then once weekly and at the end of the experiment, resulting in a total of 7 sampling days. The water samples were taken from the sump (see Figure 2.1) in the mornings, directly before the water exchange. A subsample was immediately acidified with H_2SO_4 (pH$\leq$ 2; APHA 5220, 1998) and stored at 4 °C for later analysis of the COD. Samples for the determination of total suspended solids (TSS) were taken two days before the end of the experiment and analyzed according to APHA 2540D (1998).

2.2.6 Analyses

The frozen fish samples were ground and homogenized in a mincing machine (Model TW-R 70, FEUMA Gastromaschinen GmbH, Gößnitz, Germany). The collected fecal waste samples were freeze-dried at Zirbus Technology Benelux B.V. (Tiel, The Netherlands). For sample preparation, feed and fecal waste was ground with a centrifugal grinding mill (Retsch/Brinkmann ZM 100 /w 1.1 mm sieve, Verder NV, The Netherlands). Proximate composition of fish, feed and feces were determined according to ISO-standard analysis for determination of dry matter (DM; ISO 6496, 1983), crude ash (ISO 5984, 1978), acid insoluble ash (AIA; ISO 5985, 1981), crude fat (ISO 6492, 1999), crude protein (ISO 5983, 1997, crude protein= Kjeldahl-N x 6.25), energy (ISO 9831,1998), crude fiber (NEN/ISO 6865 using fibercap with pore diameter 23 µm), neutral detergent fiber (NDF; ISO 16472, 2006), acid detergent fiber (ADF; NEN/ISO 13906), acid detergent lignin (ADL; NEN/ISO 13906 using P2 crucibles with pore diameter 40-100 µm) and starch (NEN/ISO 15914). Sugar monomers of hemicellulose and other carbohydrates were determined after hydrolysis with hydrochloric acid in feed and feces with HPAE-PAD (High Performance Anion Exchange chromatography with Pulsed Amperometric Detection). Hemicellulose monomer content of the collected waste was calculated as the sum of xylose, mannose, arabinose, galactose, rhamnose and fucose (Saha, 2003). Cellulose content was calculated as ADF-ADL. We used biochemical oxygen demand (BOD) as an approximation for COD degradability in the fecal waste samples. BOD was determined at 5 and 10 days at a temperature of 20 °C, using a continuous manometric

measurement (BODTrak, Hach Lange, Tiel, The Netherlands). Dried and ground fecal waste samples were suspended in well water (~430 mg/L), which was inoculated with system water from a RAS used for catfish husbandry at "De Haar Vissen" (1% v/v). The samples were tested in 3 runs, each run consisted of one HNSP fecal waste sample, one LNSP fecal waste sample and a negative control containing only the water with inoculum. Each RAS got tested once, giving a total of 3 samples for each diet. Total oxygen consumption of all samples was corrected for the negative control of the respective run. Water quality was measured in the tank effluent in the solids collector twice a day before feeding. Oxygen, pH, temperature and conductivity were measured with handheld meters (WTW multi 340i and respective WTW probes, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). The analytical water samples for COD and N were analyzed with an autoanalyzer (SAN Plus, Skalar, Breda, The Netherlands) for total ammonia nitrogen (TAN = NH_3-N + NH_4^+-N), (Skalar protocol number. 155-006 w/r), NO_2-N (Skalar protocol number 467-003) and NO_X-N (Skalar protocol number 461-318). NO_3-N was calculated as NO_X-N - NO_2-N. The stored acidified water samples were analyzed for total COD (APHA 5220 D, 1998). Water exchange was measured daily with water meters (0.0001 m^3, Flodis, Schlumberger, Dordrecht, The Netherlands), which were connected to the water supply of each replicated RAS.

2.2.7 Calculations and statistics

The calculations are summarized in Table 2.2. Nutrient concentrations in the system water were assumed to change linearly between the weekly sampling days. The daily concentration estimates were used to determine the amount of nutrients discharged at a given day. The COD for starch, cellulose and hemicellulose monomers was calculated as stoichiometric oxygen demand (Tchobanoglous et al., 2004). Mean values, standard deviations and slopes were calculated using Microsoft Excel 2010. Mean values were compared between treatments using one-way ANOVA, homogeneity of variance was tested with Levene's test ($p > 0.05$). Parameters which violated the required homogeneity of variance were compared with a two-sided t-test, accounting for unequal variance (IBM SPSS Statistics, version 19).

Table 2.2: Calculations.

Parameter	Unit	Formula
Fish performance		
Fish growth (G)	[g]	$G=W_{final}-W_{initial}$
Feed conversion ratio (FCR)	[g/g]	FCR=FC/G
Specific growth rate (SGR)	[%W/d]	$SGR=((\ln W_{final}-\ln W_{initial})/T) * 100\%$
Solid waste production, recovery and composition		
Apparent digestibility (ADC)	[%]	$ADC_X=(1-AIA_{feed}/AIA_{feces} * X_{feces}/X_{feed}) * 100\%$
Solid waste production (FL)	[g/kg feed]	$FL = 1000\ g * (100\%-ADC_{DM})/100\%$
Solid waste recovery (RR)	[%]	RR = SC/FL * 100%
Nitrogen-free extracts (NFE)	[g/kg DM]	NFE = DM-CP-CF-CA
Non-starch polysaccharides (NSP)	[g/kg DM]	NSP= NFE-St
COD in feed and feces, based on energy (COD_E)[a]	[g COD/g DM]	$COD_E = E/(3.40\ kcal/g\ O_2)$
COD crude protein (COD_{CP})[a]	[g COD/g DM]	$COD_{CP} = 1.66\ g\ O_2/g\ CP$
COD crude fat COD_{CF})[a]	[g COD/g DM]	$COD_{CF} = 2.78\ g\ O_2/g\ CF$
COD starch (COD_{St})[b]	[g COD/g DM]	$COD_{St} = 1.19\ g\ O_2/g\ St$
COD cellulose (COD_C)[b]	[g COD/g DM]	$COD_C = 1.19\ g\ O_2/g\ C$
COD hemicellulose (COD_{HC})[b]	[g COD/g DM]	$COD_{HC} = (Xy+Ma+Ar+Ga) * 1.07\ g\ O_2/g + (Rh + Fu) * 1.27\ g\ O_2/g$
COD lignin & remainder (COD_{LR})	[g COD/g DM]	$COD_{LR} = COD_E-COD_{CP}-COD_{CF}-COD_{St}-COD_C-COD_{HC}$
COD NSP (COD_{NSP})	[g COD/g DM]	$COD_{NSP} = COD_C + COD_{HC} + COD_{LR}$
Solid COD degradability (COD_{Deg})	[%]	$COD_{Deg} = BOD_{10}/COD_E * 100\%$
Nutrient balances		
Nutrient mass balance on fish level	[g]	$X_{FC} = X_{RT} + X_{FL} + X_{NFL}$
Nutrient mass balance on system level	[g]	$X_{FC} = X_{RT} + X_{Acc} + X_{Col} + X_{Dis} + X_{UL}$ $X_{Acc} = X_{sys\_final} - X_{sys\_initial}$ $X_{Dis} = \sum_{t=1}^{42d}(C_{Xt}*V_t)$
Nutrient concentration between sampling days (C_t)	[g/L]	$C_{Xt} = (C_{wj}-C_{wi})/(t_{wj}-t_{wi}) * (t-t_{wi}) + C_{wi}$
Suspended/dissolved nutrient production in system (X_{Pro})	[g]	$X_{Pro} = X_{Acc} + X_{Dis}$
Nitrogen system load (N_{SLD})	[g]	$N_{SLD}= N_{NFL} + N_{FL} * (100\%-RR)/100\%$
COD system load (COD_{SLD})	[g]	$COD_{SLD} = COD_{FL} * (100\%-RR)/100\%$
System losses (X_{SLS})	[g]	$X_{SLS} = X_{SLD} - X_{Pro}$
COD/N ratios		
COD/N in metabolic waste (COD/N_{MW})	[g/g]	$COD/N_{MW} = COD_{FL}/N_{NFL}$
COD/N biofilter load (COD/N_{BF})	[g/g]	$COD/N_{BF} = COD_{SLD}/N_{SLD}$
COD/N system output (COD/N_{SO})	[g/g]	$COD/N_{SO} = (COD_{FL} * RR/100\%)/N_{Pro}$
Bioavailable COD/N in system output (COD_{Deg}/N_{SO})	[g/g]	$COD_{Deg}/N_{SO} = (COD_{FL} * COD_{Deg}/100\% * RR/100\%)/N_{Pro}$

[a] Henken et al., 1986; [b] stoichiometric oxygen demand calculated acc. to Tchobanoglous et al. (2004), St: Starch: $n[C_6H_{10}O_5]$, C: cellulose: $n[C_6H_{10}O_5]$, Xy: xylose ($C_5H_{10}O_5$), Ma: mannose ($C_6H_{12}O_6$), Ar: arabinose ($C_5H_{10}O_5$), Ga: galactose ($C_6H_{12}O_6$), Rh: rhamnose ($C_6H_{12}O_5$), Fu: g fucose ($C_6H_{12}O_5$); W: fish weight, initial and final, FC: feed consumed (g DM), T: # feeding days (41 d), X: nutrient, AIA: acid insoluble ash, SC: solid waste collected (g/kg feed) DM: dry matter, CP: crude protein, CF: crude fat, CA: crude ash, E: energy in kcal/g DM, BOD_{10} in g O_2/g DM, RT: retention, FL: fecal loss, NFL: non-fecal loss, Acc: accumulated in system volume, Col: collected as solid waste, Dis: discharged, UL: unexplained losses, C: concentration (g/L), V: discharged volume (L), w_i: sampling day of a given week, w_j: sampling day after w_i, t: day between w_i and w_j

2.3 Results

2.3.1 Water quality and fish performance

No significant differences in average water quality were observed between the treatments (Table 2.3). Conductivity was maintained between 905 and 2230 µS/cm in all systems during the experiment. The average pH was 7.3 and maintained between 6.9 and 7.8 for all systems. Oxygen values were always above 7.9 mg/l during the measurements. Maximal observed TAN, NO_2-N and NO_3-N concentrations were 0.28 mg/l, 0.47 mg/l and 64.1 mg/l, respectively. TSS levels measured two days before the end of the experiment were 1.0 ± 0.4 and 1.6 ± 0.8 mg/L for HNSP and LNSP, respectively, and showed no significant differences between treatments (p= 0.285). The sodium bicarbonate consumption in the LNSP systems was slightly higher than in HNSP systems (p= 0.040). Fish survival was 100% and no feed refusal occurred during the experiment. Fish of the HNSP treatment showed better growth performance when compared with LNSP fish, resulting in a significantly lower feed conversion ratio (FCR) for HNSP (p= 0.001, Table 2.4).

Table 2.3: Water quality and management (mean ± standard deviation, n=3).

Parameter	Unit	HNSP	LNSP	*p*
Daily measurements				
Temperature	[°C]	15.7 ± 0.1	15.6 ± 0.0	0.076
Oxygen	[mg/L]	9.0 ± 0.0	9.0 ± 0.0	0.597
Conductivity	[µS/cm]	1529.4 ± 32.1	1492.7 ± 9.5	0.130
Weekly measurements				
TAN	[mg/L]	0.13 ± 0.0	0.11 ± 0.0	0.053
NO_2-N	[mg/L]	0.24 ± 0.0	0.24 ± 0.0	0.896
NO_3-N	[mg/L]	51.7 ± 2.6	49.4 ± 1.1	0.222
tCOD	[mg/L]	23.0 ± 2.0	22.6 ± 2.6	0.830
System management				
Water exchange	[L/kg feed]	453.9 ± 1.6	453.9 ± 1.8	0.992
Sodium bicarbonate addition	[g/kg feed]	125.2 ± 2.2	129.9 ± 1.7	0.040

Table 2.4: Fish performance (mean ± standard deviation, n=3). FCR: Feed conversion ratio, SGR: Specific growth rate.

Parameter	Unit	HNSP	LNSP	*p*
Initial Individual Weight	[g]	100.9 ± 3.2	105.6 ± 4.6	0.224
Final Individual Fish Weight	[g]	192.3 ± 2.4	183.9 ± 3.6	0.030
Survival	[%]	100.0 ± 0.0	100.0 ± 0.0	n.a.
FCR	[kg/kg]	0.9 ± 0.0	1.1 ± 0.0	0.001
SGR	[%/d]	1.6 ± 0.1	1.4 ± 0.1	0.011

2.3.2 Composition of feed and feces, apparent digestibility and recovery

Proximate composition of feed and feces are presented in Table 2.5, the calculated apparent digestibility can be found in Table 2.6. The main difference between our experimental diets when compared to commercial diets is a relatively high NFE-fraction of 36-43%, which was necessary to generate the contrast in NSP. NFE contents in commercial diets used by Dalsgaard and Pedersen (2011) were between 10-15%. Digestibility of crude protein and ash was similar for both diets. Fat and starch digestibility were significantly higher for the HNSP diet (p= 0.005 and p< 0.001, respectively). The negative ADC for NSP in LNSP is not significantly different from zero (One sample t-test, p= 0.873). Changing the composition of dietary nitrogen-free extracts (NFE) from starch to NSPs significantly decreased NFE digestibility for the HNSP diet, which ultimately reduced dry matter (DM) digestibility of HNSP (p< 0.001). Based on DM digestibility, the HNSP diet produced ~40% more solid waste when compared with LNSP. Furthermore, a higher recovery of HNSP feces doubled COD output from HNSP systems when compared with LNSP systems (Figure 2.2). NSPs constituted almost two thirds of the COD fraction of HNSP feces, whereas it only contributed with ~5% to the COD in LNSP feces (Table 2.7). Hemicellulose and cellulose made up almost 40% of the COD in HNSP feces. Starch was with ~40% the biggest COD fraction in LNSP feces. COD biodegradability based on biochemical oxygen demand was significantly higher in LNSP feces when compared with HNSP feces (Figure 2.3).

Table 2.5: Feed and feces composition (mean ± standard deviation, n=3).

		Feed[a]		Feces[b]		
Parameter	**Unit**	**HNSP**	**LNSP**	**HNSP**	**LNSP**	***p***
Dry matter	[g/kg FW]	980.9	979.3			
Crude protein	[g/kg DM]	412.0	396.3	141.6 ± 3.5	197.7 ± 12.4	0.002
Crude fat	[g/kg DM]	134.6	87.6	31.7 ± 0.8	37.6 ± 1.6	0.005
Ash[c]	[g/kg DM]	94.4	81.7	230.0 ± 2.5	280.3 ± 9.9	0.009[e]
NFE[d]	[g/kg DM]	359.0	434.4	596.7 ± 5.6	484.4 ± 19.9	0.001
Starch	[g/kg DM]	166.2	415.6	43.2 ± 0.6	325.9 ± 19.0	0.001[e]
NSP	[g/kg DM]	192.8	18.8	553.5 ± 5.1	158.5 ± 13.4	0.000
Gross energy	[kJ/g DM]	21.2	20.1	15.2 ± 0.2	14.0 ± 0.1	0.002
Crude fiber	[g/kg DM]	54.9	1.8	231.9 ± 6.3	21.3 ± 1.7	0.000
AIA	[g/kg DM]	17.6	17.1	76.6 ± 0.9	104.0 ± 4.3	0.000

[a] n=1, [b] n=3, mean ± standard deviation, [c] Ash including AIA, [d] Nitrogen-free extract, including crude fiber, [e] Unequal variance, calculated with t-test, FW: fresh weight, DM: dry matter.

Table 2.6: Apparent digestibility and fecal waste recovery (mean ± standard deviation, n=3).

Parameter	**Unit**	**HNSP**	**LNSP**	***p***
Dry matter	[%]	77.0 ± 0.3	83.5 ± 0.7	0.000
Crude protein	[%]	92.1 ± 0.2	91.8 ± 0.4	0.314
Crude fat	[%]	94.6 ± 0.1	92.9 ± 0.5	0.005
Starch	[%]	94.0 ± 0.1	87.1 ± 1.0	0.000
Ash	[%]	43.9 ± 0.2	43.5 ± 0.7	0.430
NFE	[%]	61.7 ± 0.7	81.6 ± 1.5	0.000
NSP	[%]	33.9 ± 1.3	-39.6 ± 17.0	0.002
COD	[%]	83.5 ± 0.3	88.5 ± 0.6	0.000
Recovery (DM)[a]	[%]	78.9 ± 1.7	56.0 ± 0.5	0.000

[a] Expressed as % undigested dry matter recovered.

Table 2.7: Qualitative COD composition in fecal waste (mean ± standard deviation, n=3).

Parameter	**Unit**	**HNSP**	**LNSP**	***p***
Crude protein	[%]	22.0 ± 0.2	33.4 ± 2.3	0.001
Crude fat	[%]	8.3 ± 0.2	10.6 ± 0.4	0.001
Starch	[%]	4.8 ± 0.1	39.3 ± 2.2	0.001[a]
NSP	[%]	64.9 ± 0.2	16.7 ± 1.5	0.000[a]
Cellulose	[%]	21.6 ± 0.4	3.3 ± 0.5	0.000
Hemicellulose	[%]	17.2 ± 0.2	6.5 ± 0.2	0.000
Lignin & remainder	[%]	26.1 ± 0.4	7.9 ± 2.2	0.000

[a] Unequal variance, calculated with t-test

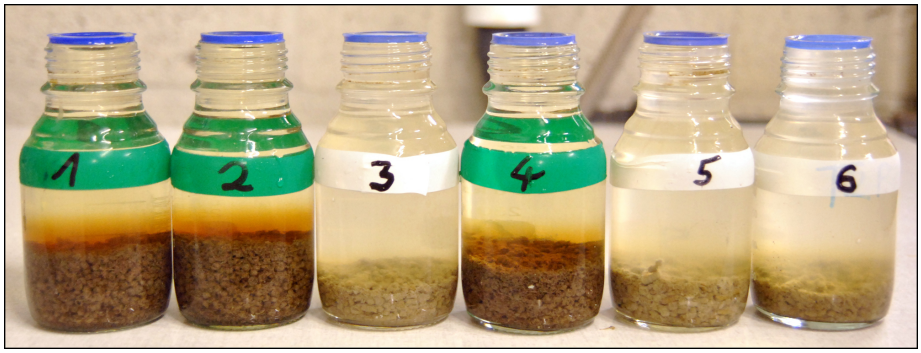

Figure 2.2: Morning collection of fecal waste during the experiment. Bottles with the numbers 1, 2 and 4 were connected to HNSP systems. Bottles 3, 5 and 6 were connected to LNSP systems. Clear differences in color were observed between the supernatant of HNSP and LNSP feces, which were as well reflected in the water color of the respective RAS.

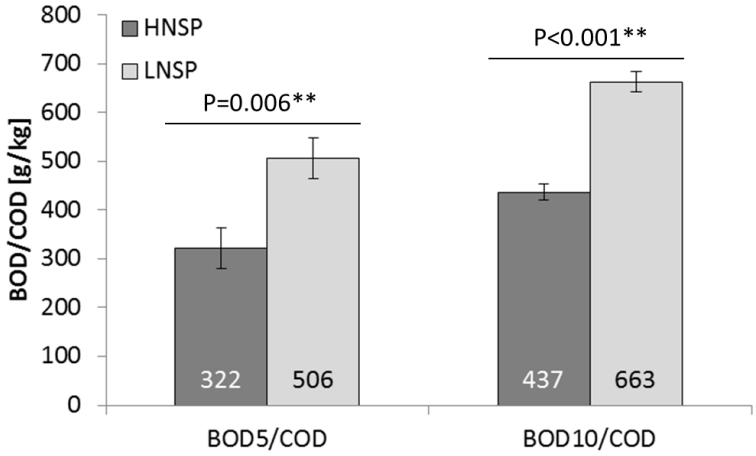

Figure 2.3: BOD/COD-ratios of recovered fecal waste (mean ± standard deviation, n=3).

2.3.3 Waste production on fish and system level

The absolute nitrogen retention in fish per kg feed was significantly higher in HNSP treatments than in LNSP (p= 0.015; Table 2.8). However, the calculated relative digestible nitrogen retention was 52.1 ± 0.6% and 48.8 ± 2.2% for HNSP and LNSP, respectively, indicating a possible trend for higher N retention in HNSP (p= 0.064). Branchial and urinary nitrogen excretion (BUN) was ~29 g N/kg feed and not significantly different between treatments (p= 0.369). Measured dissolved nitrogen production on system level was ~27 g N/kg feed and not significantly different between treatments (p= 0.928). COD retention in fish was significantly higher for HNSP when compared with LNSP (p= 0.011). Consequently, the respiratory losses, which were calculated as non-fecal loss of COD (Table 2.8), were significantly higher for LNSP when compared with HNSP (p= 0.006). HNSP-fed fish produced ~50% more fecal COD when compared with fish of the LNSP treatment. Taking recovery into account, solid COD output from HNSP systems was twice as high as in LNSP systems. Dissolved/suspended COD production on system level was not significantly different between treatments (p= 0.917).

2.3.4 System load and system losses

Nitrogen system loads were not significantly different between treatments (p= 0.062; Table 2.8). Nitrogen system losses were significantly higher for LNSP when compared with HNSP (p= 0.048). However, the relative N losses were not significantly different between treatments (10.5 ± 2.0% for HNSP and 16.1 ± 2.9% for LNSP; p= 0.052). Although the COD system load was significantly lower in HNSP systems (p= 0.005), the COD losses were significantly higher in LNSP systems (p= 0.002). The ratio of

Table 2.8: COD and N production on fish and system level, expressed per kg of feed dry matter (DM; mean ± standard deviation, n=3).

	COD [g/kg feed DM]			N [g/kg feed DM]		
Parameter	**HNSP**	**LNSP**	***p***	**HNSP**	**LNSP**	***p***
Feed	1491.0	1413.4	n.a.	65.9	63.4	n.a.
Retention	528.8 ± 18.7	434.1 ± 31.4	0.011	31.6 ± 0.3	28.4 ± 1.4	0.015
Respiration[a]	716.2 ± 18.2	816.8 ± 27.4	0.006	-	-	-
Solid waste						
Fecal loss	246.0 ± 3.9	162.5 ± 8.3	0.000	5.2 ± 0.1	5.2 ± 0.3	0.953
Recovery	194.0 ± 4.5	91.0 ± 4.6	0.000	4.1 ± 0.1	2.9 ± 0.2	0.001
Suspended/dissolved						
tCOD in system	13.2 ± 1.1	13.3 ± 1.6	0.917	-	-	-
BUN	-	-	-	29.1 ± 0.4	29.8 ± 1.2	0.369
N in system	-	-	-	27.0 ± 0.8	26.9 ± 0.5	0.928
System						
Load	52.0 ± 4.4	71.5 ± 3.9	0.005	30.2 ± 0.3	32.1 ± 1.3	0.062
Loss	38.8 ± 3.3	58.2 ± 2.9	0.002	3.2 ± 0.6	5.2 ± 1.1	0.048

[a] Assumed that all NFL-COD is due to respiration, BUN: branchial and urinary nitrogen losses.

dissolved COD remaining in the system and the COD system load can be used as a crude indicator for the digestibility of the COD system load within the RAS. The calculated "digestibility" of the COD system load was 74.6 $\pm$ 0.2% for HNSP and significantly lower when compared with LNSP (81.4 $\pm$ 1.6%, p= 0.002).

2.3.5 COD/N-ratios

The COD/N-ratios in the feeds were ~22 g COD/g N and similar for both diets. Solid COD/dissolved N ratios in the metabolic waste and system output were always higher for HNSP when compared with LNSP (Figure 2.4). However, the comparably low COD degradability of HNSP feces severely decreased the bioavailable COD/N ratios in the system output. LNSP had a significantly higher COD/N ratio in the system load when compared to HNSP (2.2 $\pm$ 0.2 and 1.7 $\pm$ 0.2, respectively; p= 0.029). The effective load of degradable organic matter expressed as BOD_5/N (Zhu and Chen, 2001) was 0.6 $\pm$ 0.1 and 1.1 $\pm$ 0.1 for HNSP and LNSP, respectively (p= 0.002).

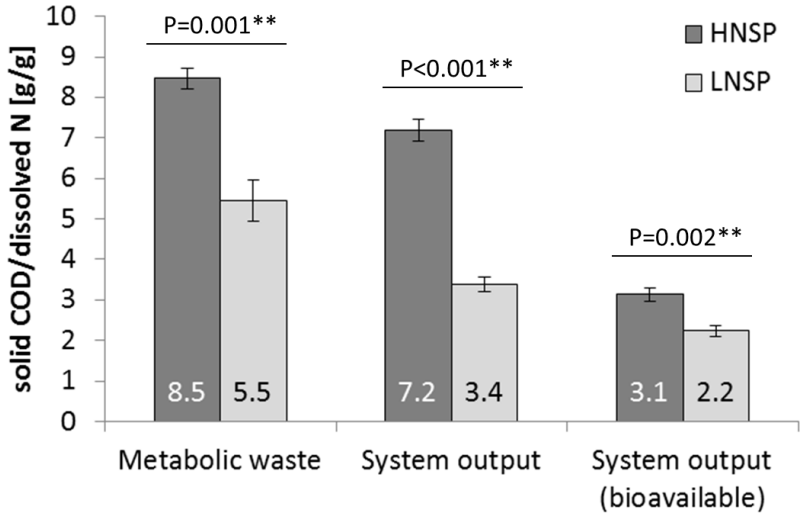

Figure 2.4: COD/N ratios for denitrification in metabolic waste of fish and system output, determined as solid COD/dissolved N taking system losses and bioavailability of the collected solids into account (mean ± standard deviation, n=3).

2.4 Discussion

2.4.1 Water quality and fish performance

Water quality was within recommended limits for rainbow trout in general, the exception were elevated nitrite levels (Colt, 2006; Timmons and Ebeling, 2007). Nitrite toxicity was counteracted from the beginning of the experiment by maintaining chloride:nitrite ratios above 10:1 (w/w; Colt, 2006), and no negative effects on fish performance were observed. Feed conversion was comparable with literature (Gomes et al., 1995; Pedersen et al., 2012). The supply of digestible energy was not significantly different between treatments (data not shown). The lower growth for the LNSP diet could be explained by a ~4% lower protein supply and decreased fish performance due to a high starch content in the LNSP diet (Hemre et al., 1995). However, the observed difference in FCR of 15% has only a minor effect on nitrogen retention and does not significantly contribute to the observed contrasts in COD/N-ratios.

2.4.2 Feed composition and apparent digestibility

The protein content of the diets was ~40%, which is lower than the 45-48% protein commonly used in commercial feeds. Given the large contrasts in COD production and recovery between diets, the 10% lower N content in LNSP feed has little influence on

COD/N ratios. The determined digestible protein/digestible energy ratios of approximately 21 kJ/g were close to the recommendations for rainbow trout of 22-24 kJ/g (Kaushik and Médale, 1994). The crude fiber content in the HNSP diet was 5.5%, which is still comparable to some commercial diet formulations with crude fiber contents up to 5%. The starch content of ~42% in the LNSP diet was unusually high but necessary to ensure a maximal contrast in NSP between diets (Hemre et al., 1995). Hemre et al. (1995) have shown that dietary starch levels of $\geq$ 22% have an adverse effect on nutrient digestibility and growth performance in salmonids. Accordingly, we observed a significantly lower starch and fat digestibility for the LNSP treatment. The contrast in digestible energy allocation in diets resulted in a ten times higher dietary NSP content in HNSP when compared with LNSP. The high inclusion level of NSPs in the HNSP diet resulted in a significantly lowered DM-digestibility of 77%. This is comparable to the theoretical values of Bureau and Hua (2010), who reported an estimated dry matter digestibility of 78% for trout fed different commercial diets. The dry matter digestibility for LNSP of 84% is probably closer to recent highly digestible and nutrient-dense commercial diets. Despite the fact that digestibility was estimated indirectly with a marker using settled solids in RAS, the overall results seem comparable to ADC values reported in literature (Dalsgaard and Pedersen, 2011; Roque d'Orbcastel et al., 2008). Although this method of digestibility determination has been accepted in the past (Farhangi and Carter, 2007; Saravanan et al., 2012; Thiessen et al., 2003), we cannot exclude the possibility that the original feces composition could have been changed by biomass originating from RAS. However, we believe that the contribution of non-fecal solids is only minor and would not affect the validity of the study.

2.4.3 Nitrogen balance

The formulated diets performed well and resulted in similar nitrogen production rates on fish and system level. Nitrogen production calculated as BUN was approximately 30 g/kg feed for both diets, only growth and nitrogen retention was slightly higher for HNSP. The observed nitrogen retention of 45-48% and excretion as BUN of 46% corresponds well with the results of Dalsgaard and Pedersen (2011), who measured 45.6% and 47.6% for N retention and suspended/dissolved N production, respectively. Nitrogen from not recovered feces contributed only 3.6% and 7.1% to the nitrogen in the system load for HNSP and LNSP, respectively. Although LNSP showed only a trend towards a higher N load, the nitrogen system losses were significantly higher in LNSP systems. This is most likely the consequence of a higher COD load in LNSP systems, promoting assimilatory or dissimilatory nitrogen removal (van Rijn et al., 2006).

2.4.4 COD production and recovery

COD production based on digestibility was significantly increased for RAS using the HNSP diet. Waste production as dry weight was increased by 50% for HNSP when compared with LNSP. Our results demonstrate the importance of the proposed general guidelines for reducing nutrient emissions from aquaculture by formulating highly digestible and nutrient-dense diets (Cho and Bureau, 1997). However, HNSP showed an unexpected high recovery, even outperforming the observed recovery rate of 71% in direct sedimentation within the fish tank of Dalsgaard and Pedersen (2011). The observed 56% recovery of LNSP feces was comparable to literature, reported recovery rates for swirl separators were 63% (Couturier et al., 2009), radial-flow settler 48% (Summerfelt and Penne, 2005) and 60 $\pm$ 28% in sedimentation (Roque d'Orbcastel et al., 2008). The increase of the recovery rate in HNSP systems is most likely caused by increased cohesion of fecal pellets, resulting in increased particle size, higher sedimentation rate and lower leaching rates (Brinker et al., 2005a). The significantly higher recovery rate of HNSP feces, combined with the lower COD digestibility, resulted in a two times higher COD output from HNSP systems. Our study shows that internal NSPs originating from the used plant ingredients increased recovery rate by 40%. Brinker et al. (2005b) demonstrated that the inclusion of guar gum as a binder in trout diets increased solids removal in drum filtration by 40%. The increase in fecal recovery observed in our experiments suggests that an addition of external NSPs as binders could become obsolete if the right feed ingredients are chosen (Amirkolaie et al., 2005; Brinker et al., 2005a). This offers an interesting, new perspective for the feed formulation process.

2.4.5 COD/N load and system performance

Another consequence of the increased COD recovery was a significantly lower COD load on the biofilters in HNSP systems. Even with a significantly higher COD production in HNSP systems, the biofilters were loaded with less COD than LNSP systems. Zhu and Chen (2001) reported that COD/N ratios below 2.86 g COD/g N or 1.76 g BOD_5/g N (C/N=1) are favorable for the nitrification performance of biofilters in RAS. Assuming that all produced nitrogen would be converted to NO_3-N, the COD/N ratios in the system load would have been 1.7 $\pm$ 0.2 and 2.2 $\pm$ 0.2 for HNSP and LNSP, respectively. However, the BOD_5/N-ratio of 0.6 in HNSP treatments was almost 50% lower than the BOD_5/N ratio of LNSP, indicating that high NSP diets could be beneficial for the nitrification performance of biofilters in RAS. Zhu and Chen (2001) estimated that a typical BOD_5/N ratio in RAS would be around 4, thus the HNSP diet would represent indeed a diet with a remarkably low BOD_5 load on the biofilters. Since our biofilters were designed with an ample safety margin, no apparent adverse effect of LNSP diet on nitrification performance could be observed. However, if a system is operated close to its limit, diet formulation could become a critical point in maintaining sufficiently high nitrification capacity.

2.4.6 COD/N ratios and COD bioavailability for denitrification

Our experiment has clearly shown how diet composition can be used to manipulate COD/N ratios along the feed-fish-waste axis. The main factors influencing COD/N ratios were COD digestibility and recovery. The COD-N ratios available for denitrification in the system output of HNSP and LNSP systems would be 7.2 and 3.4, respectively. Taking BOD_{10}/COD ratios into account, the degradable COD/N-ratios in the output of HNSP and LNSP systems would drop to 3.1 and 2.2, respectively. However, since BOD_{10} was not determined in fresh samples, COD bioavailability could have been underestimated due to effects of drying or insufficient inoculation (Grous et al., 1986; Pagga, 1997). Klas et al. (2006a) showed that a single-sludge denitrification reactor using only internal COD sources could operate at a COD/NO_3-N ratio of 4.5, or 3.8 degradable COD/N if the reported degradability of 84% is taken into account (Klas et al., 2006b). Although the HNSP diet produced sufficient COD, none of the experimental diets could provide sufficient degradable COD for full denitrification. In-depth analysis of the fiber composition revealed that 65% of the HNSP-COD was allocated in fibers, of which cellulose and hemicellulose make up almost 40% of the total COD. Fibers, especially lignocellulosic material, are considered as a poorly degradable carbon source (Hendriks and Zeeman, 2009). If full denitrification is hampered by limited COD degradability, a prolonged sludge retention time could increase the amount of available COD (Noike et al., 1985). An alternative strategy to improve COD bioavailability could be the addition of exogenous enzymes to facilitate fiber degradation (Pérez et al., 2002).

2.5 Conclusions

Our results clearly show how manipulating dietary carbohydrate composition can affect waste production and composition in RAS. NSPs originating from plant ingredients significantly decreased COD and dry matter digestibility and drastically increased COD output from RAS. Furthermore, NSPs originating from plant ingredients increased fecal recovery. Although diets rich in NSP can potentially produce more COD, the effective COD load on the biofilters can be lower compared to a low NSP diet. The increased COD production and recovery of high NSP diets could be exploited by using the excess COD as an internal carbon source for denitrification. Complete nitrogen removal could be possible for the tested HNSP diet, however, it is not clear if this process would be hampered by a low degradability of the COD. The major factor reducing COD degradability is most likely a high content of poorly degradable lignocellulosic material. More research is needed to investigate the role of fibers as a carbon source for denitrification and how limited COD bioavailability could be counteracted.

2.6 Acknowledgements

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3 Effects of diet composition and ultrasound treatment on particle size distribution and carbon bioavailability in feces of rainbow trout

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Abstract

Fibers originating from unpurified plant protein sources could impose new challenges on the treatment of solid waste in RAS. Fibers, often expressed as non-starch polysaccharides (NSPs), have a high structural integrity and low digestibility in fish. Thus, changing diet composition could change the particle size distribution and subsequent recovery of feces with microscreens in RAS. Furthermore, fibers can hamper carbon bioavailability in fecal waste, which could become a problem if feces are going to be used as an endogenous carbon source for denitrification in RAS. The goal of this experiment was to investigate if differences in diet formulation can affect particle size distribution and microscreen recovery in fecal waste of rainbow trout (*Oncorhynchus mykiss*). Furthermore, we investigated the potential of ultrasound treatment to break down particles and increase carbon bioavailability in feces. A high NSP (HNSP) and low NSP (LNSP) diet was used to generate a contrast in fecal fiber content. Feces were collected in four flow-through tanks, of which two tanks were fed the HNSP diet and two the LNSP diet. The collected feces from each tank were sonicated in duplicate with high-intensity, low-frequency ultrasound at five different energy levels (f= 20 Hz, 0.6 W/ml, t= 0, 0.25, 1, 4, and 16 min). The particle size distribution of the treated samples was measured by wet sieving and TSS (1000, 500, 200, 100, 63, 36, 1.2 µm screen size). Carbon bioavailability in sonicated fecal waste samples was determined with oxygen uptake rate (OUR) tests. Cumulative particle size distributions showed that HNSP feces contained more particulate material, and bigger particles, when compared with LNSP feces. Almost 50% of HNSP feces could have been recovered on a microscreen of 36 µm after wet sieving, whereas it was only 10% for LNSP feces. However, the production of small particles (1.2- 36 µm), which could pass a drum filter screen and potentially accumulate in RAS, was approximately 50 g/kg feed for both diets (p= 0.913). Carbon bioavailability was almost three times higher in untreated LNSP feces when compared with HNSP feces. Although ultrasound treatment resulted in a small but significant increase of dry matter below 36 µm (p= 0.015), it had no significant effect on the median particle size (p= 0.098). Ultrasound treatment increased carbon bioavailability by 7-10%, the effect was significant only for the HNSP feces (p= 0.037). These results suggest that the use of unpurified plant-based ingredients can increase particle size in feces and increase solid waste recovery with microscreens in RAS. Ultrasound treatment does not seem to be an effective method to increase short-term bioavailability in feces of rainbow trout, since fecal particles withstood up to 20,000 kJ/kg DM without major changes in particle size distribution.

3.1 Introduction

Modern fish feeds are being formulated with increasing amounts of alternative plant protein sources (Naylor et al., 2009). Increasing the amount of unpurified plant ingredients in feeds will introduce fibers (i.e. non-starch polysaccharides, NSPs), which are considered indigestible in fish (Lovell, 1998). Due to their low digestibility and high structural integrity, fibers could potentially change the particle size distribution of fecal waste and its subsequent recovery with microscreens in recirculating aquaculture systems (RAS). Furthermore, the low degradability of fibers could hamper the use of feces as a carbon source in denitrification (Meriac et al., 2014). Innovative RAS use the organic carbon (expressed as chemical oxygen demand, COD) originating from fecal waste for denitrification, effectively reducing water demand and nutrient emissions in RAS (Gelfand et al., 2003; Martins et al., 2009). However, the total removal of nitrate can be limited by the bioavailability of carbon (Kaiser and Schmitz, 1988; Klas et al., 2006a). To overcome a limited carbon bioavailability, low frequency, high intensity ultrasound is commonly applied as a pre-conditioning method in wastewater treatment and anaerobic digestion (Pilli et al., 2011; Tiehm et al., 1997). Several studies have shown how ultrasound can increase carbon bioavailability by decreasing particle size and COD solubilization (Tiehm et al., 1997). The goal of our research was to determine [i] if differences in diet formulation can affect the particle size distribution in feces and subsequent recovery with microscreens, and [ii] if ultrasound can break down fecal particles to increase carbon bioavailability for a waste treatment process like denitrification.

3.2 Material and methods

3.2.1 Feed and feeding

The two experimental feeds were formulated with the objective to produce feces with a contrast in fiber content between the treatments. The fish were fed a high starch, low NSP (LNSP) and a low starch, high NSP (HNSP) diet (Table 3.1). We used extruded diets, which were produced by Research Diet Services (Wijk bij Duursteede, The Netherlands). Acid insoluble ash was used as a marker to determine digestibility (Atkinson et al., 1984; Vandenberg and De La Noue, 2001). The fish were fed a floating pellet of 3 mm, and the daily ration of ~1.5% body weight/d was equally divided between morning (9:30 h) and afternoon feeding (16:30 h). Feeding was done by hand and under close observation to avoid leftover pellets in the feces collection. In case a tank would not eat the whole ration, the leftover pellets would be collected and counted. In the subsequent feeding, the ration for the remaining tanks would be reduced by the amount of uneaten feed recovered to ensure homogenous growth between tanks and diets. Feed samples were collected weekly and analyzed as a pooled sample at the end of the experiment to determine proximate composition. Dry matter digestibility was 78% and 83% for HNSP and LNSP,

respectively.

Table 3.1: Feed ingredients as ordered at the manufacturer.

Parameter	Unit	LNSP	HNSP
Proximate composition			
Dry matter	[g/kg FW]	948	964
Crude protein	[g/kg DM]	389	409
Crude fat	[g/kg DM]	92	132
Crude ash	[g/kg DM]	82	95
Nitrogen-free extracts	[g/kg DM]	417	342
NSP	[g/kg DM]	8	198
Starch	[g/kg DM]	410	143
Acid-insoluble ash	[g/kg DM]	18	19
Energy	[g/kg DM]	20.2	21.2
Ingredients			
Fish meal[a]	[g/kg FW]	410	220
Fish oil[b]	[g/kg FW]	22	102
Soy bean meal[c]	[g/kg FW]	-	180
Wheat flour[d]	[g/kg FW]	530	95
Sunflower seed meal[e]	[g/kg FW]	-	180
Rape seed meal[f]	[g/kg FW]	-	180
Monocalciumphosphate[g]	[g/kg FW]	-	5
L-Lysine HCl[h]	[g/kg FW]	4	4
DL-Methionine[i]	[g/kg FW]	4	4
Diamol[j]	[g/kg FW]	20	20
Premix[k]	[g/kg FW]	10	10

[a] TripleNine Fish Protein, Esbjerg, Denmark; [b] Coppens International, Helmond, The Netherlands; [c] Cargill, Amsterdam, The Netherlands; [d] Meneba,Weert, The Netherlands; [e] Arkervaart-Twente, Leusden, The Netherlands. [f] ADM, Spyck, Germany. [g] Tessenderlo Chemie, Rotterdam, The Netherlands; [h] Sewon Paik Kwang Industrial Co. Ltd., Jeollabuk-do, South Korea; [i] Evonik, Hanau, Germany; [j] Damolin A/S, Fur, Denmark; [k] Premix, includes vitamins,minerals and trace elements.

3.2.2 Fish husbandry

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were kept on flow-through in a total of four 130 L-tanks, each tank was equipped with a hydrocyclone for feces collection (V = 17 L, Aquaoptima AS, Trondheim Norway). The flow rate of the tanks was ~6.5 L/min, resulting in a hydraulic surface load of ~200 $m^3/m^2/d$ on the hydrocyclones. The photoperiod was set to 12:12 light/dark. Two weeks prior to the start of the experiment, each tank was stocked with 15 fish with an individual weight of ~230 g for acclimatization. The fish were weighed at stocking, before and at the end of each experimental period. The feed conversion ratio was 1.1 and 1.2 for the HNSP and LNSP diet, respectively. The

specific growth rate was 1.3% of body weight per day. The average water temperature was 15.5 °C and O_2 concentration was 6.5 mg/L for both treatments.

3.2.3 Feces collection

Feces were collected twice a day before feeding. The feces collection bottles were connected to the bottom of the hydrocyclones and cooled with ice water (Figure 3.1), similar as described in Saravanan et al. (2012). Morning and afternoon collections were pooled for each system to give a 24 h composite sample for the sonication experiments. To determine proximate composition of the fecal waste, feces were collected between the two experimental periods for five subsequent days. The collected feces were stored in aluminum trays at -20 °C for later analyses. The efficiency of fecal recovery in sedimentation was determined by relating the total amount of collected dry matter to the amount of feces produced based on dry matter digestibility of the feeds. The resulting feces recovery rate was 68% and 49% for HNSP and LNSP, respectively.

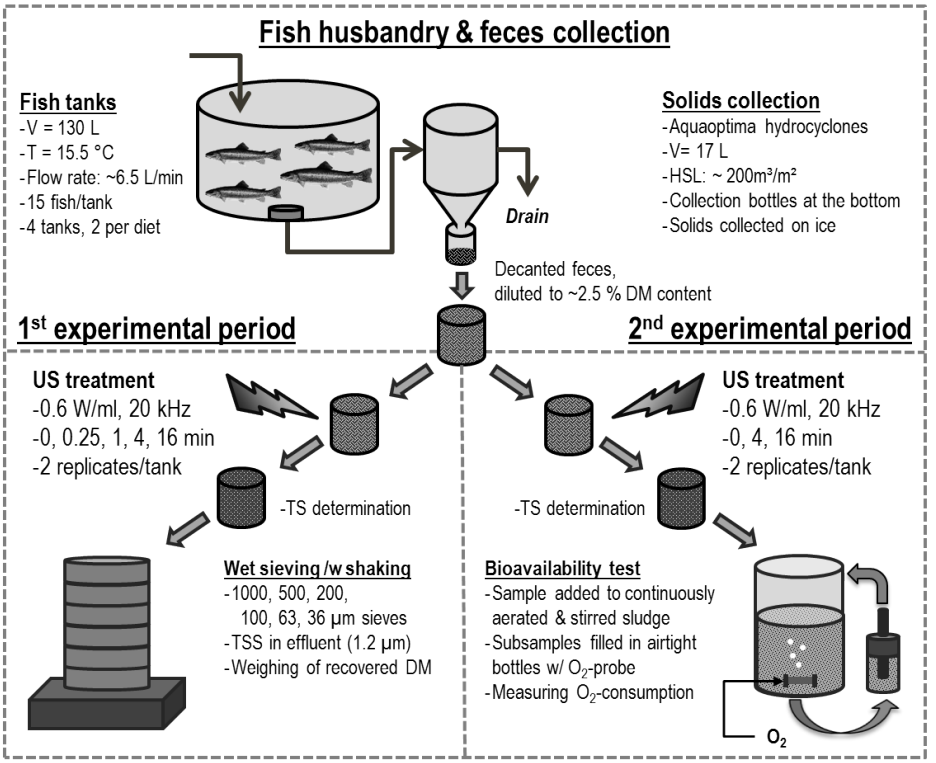

Figure 3.1: Husbandry conditions and experimental design. HSL: hydraulic surface load, US: ultrasound, TS: total solids, TSS: total suspended solids.

3.2.4 Experimental periods

In the first experimental period, we measured the differences in particle size distribution in fecal waste produced on a high fiber (HNSP) and a low fiber (LNSP) diet. Furthermore, we determined how ultrasound treatment affects the particle size distribution in HNSP and LNSP feces (Figure 3.1). In the second experimental period, we investigated the effect of ultrasound treatment on short-term carbon bioavailability in feces using an oxygen uptake rate (OUR) test. The scope of our research calls for an alternative definition and measurement of particle size. Fecal pellets will inevitably disintegrate within a upflow sludge blanket denitrification reactor due to shear forces caused by the stirring of the sludge bed. Thus, we used wet sieving as an invasive measurement to determine the size distribution of resilient particles within the fecal pellet after disintegration.

First experimental period

The fish had an initial individual body weight of ~250 g and were fed at 1.2% body weight per day during the first experimental period. The collected feces were diluted to a dry matter content of 2.5- 3% (Show et al., 2007) and subsamples of 100 ml were taken for ultrasound treatment. We used a total of four sonication times and a control without ultrasound treatment (0, 0.25, 1, 4 and 16 min). The ultrasound homogenizer was operated at a power density of 0.6 W/ml (f= 20 kHz, HD 3200 Sonopuls, Bandelin, Germany; Probe KE 76, Bandelin, Germany). After sonication, the sample was homogenized on a magnetic stirrer and sampled for total solids content (APHA 2540 B, 1998). A subsample of 50 ml (~1.5 g dry matter) was used to determine particle size distribution by serial wet sieving. We used sieves of 1000, 500, 200, 100, 63 and 36 µm mesh size in the shaker (Retsch AS200 control, Retsch, Germany). The work protocol for sieving was adapted from Oshita et al. (2004) and consisted of 3 steps: [i] Flushing while shaking for 5 min, [ii] 3 x filling while shaking, then draining (3 x 10 min), [iii] Flushing while shaking for 5 min. The sieving apparatus was shaken at an amplitude of 2 mm and approximately 40 L of water were used for flushing. The water used for flushing was collected, weighed to determine the total volume and analyzed for total suspended solids (TSS, APHA 2540 D, 1998). The material, which was retained on the sieves was washed into separate glass beakers and concentrated on pre-weight paper filters (11 µm, Ø 90 mm, No. 1, Whatman, England). The dry matter of the filters and collected material was determined similar to APHA 2540 B.

Second experimental period

The initial fish weight was 500 g and the average feeding rate was 2% of body weight. Fecal waste samples were collected and diluted similar to the first experimental period. Total solids content of the samples was determined according to APHA 2540 B (1998). Each day of measurements, the diluted feces from one tank were divided in three sub-

samples of 100 ml and sonicated for either 0, 4 or 16 minutes. Between 3-10 mL of sample were used directly in the OUR test, depending on the sample degradability and the activity of the sludge in the OUR test (Sludge/degradable substrate ratio of ~820 g COD/g rdCOD). The samples were stored on ice at all times to prevent degradation. Because ultrasound treatment has antimicrobial properties (Madge and Jensen, 2002), and the result of a BOD test heavily depends on the inoculum (Pagga, 1997), we chose to use the OUR test to measure the effects of sonication on short-term COD bioavailability. To provide a standardized microbial community for the OUR tests, we used sludge from an upflow sludge blanket denitrification reactor (27 °C), which has been in stable operation for a year with catfish under similar conditions as described in Martins et al. (2009). Since denitrifying bacteria are facultative anaerobes (van Rijn et al., 2006), the amount of easily degradable COD in a sample can be determined by the oxygen consumption resulting from the sample addition. This procedure is not intended to provide information on the actual denitrification potential of the sample, but to estimate the amount of easily degradable COD present. Approximately 2.5 L of sludge was harvested the day before each measurement, and incubated overnight in a water bath of 27 °C under constant aeration and stirring (250 rpm). Two hours before the test, N-allylthiourea (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) was added at a concentration of 20 mg/L to inhibit nitrification (Xu and Hasselblad, 1996). Total suspended solids and volatile suspended solids in the sludge were determined according to APHA 2540 D/E (1998). Oxygen uptake was determined in a fixed sludge volume of 2 L (main incubation) with an adapted batch test procedure of Xu and Hasselblad (1996) at a temperature of 27 °C. Oxygen concentrations were measured with WTW Oxi 340i handheld meters, using CellOx 325 probes (WTW GmbH, Weilheim, Germany), and recorded with a PC via serial interface at 10s-intervals. Endogenous oxygen consumption of the sludge was determined before sample addition. After the sample addition, the incubation was left stirring for 2 minutes to ensure homogenization and oxygen consumption was measured until the oxygen uptake rate was back to endogenous conditions. Acetate was added at a sludge/substrate ratio of ~390 g COD/g COD to determine the COD biomass yield in each new batch of sludge. The average yield was 69.3 ± 2.4% throughout the experimental period, which is comparable to 67% yield used by (Kappeler and Gujer, 1992). O_2 consumption was measured in airtight 250 ml glass bottles filled with sludge from the main incubation. O_2 concentrations in the measurement bottles were always kept above 1 mg/L. When O_2 concentrations dropped below 3 mg/L, a second bottle was filled with sludge from the main incubation and the measurement was switched to the second bottle after equilibration of the oxygen probe. This procedure allowed for an almost continuous measurement of oxygen consumption representative for the main incubation. After the switch, the sludge from the first bottle was poured back into the main incubation. The measurement bottles were kept stirred at 250 rpm in the same water bath as the main incubation.

3.2.5 Calculations and statistics

The calculations used are summarized in table 3.2. Dry matter below 1.2 µm and dissolved material was estimated indirectly in the sieving experiments by subtracting the sum of recovered dry matter from total dry matter supplied. Normality and homogeneity of variance of the data was verified using the Shapiro-Wilk test and the Levene's test, respectively (p> 0.05). Fecal waste composition was compared using a t-test, assuming unequal variances (n= 2). The effect of diet on particulate dry matter content, dry matter median, particle size median, microparticle production and initial COD degradability was analyzed using one-way ANOVA. The effect of sonication time and diet composition on particle size distribution was analyzed using a two-way ANOVA, testing for an effect of diet (D), sonication time (T), and an interaction effect (D x T). The data required $\log_{10}$-transformation before analysis with a two-way ANOVA. Data of carbon bioavailability did not comply with the criteria of homogeneity of variance (p= 0.042; Levene's test), even after transformation. Therefore, the effect of ultrasound on carbon bioavailability was analyzed by a non-parametric Kruskal-Wallis H-test. All statistical analyses were done with IBM SPSS statistics, version 20.

Table 3.2: Calculations.

W_{Tot}: total fish weight, n: number of fish in tank (constant over experimental period, since survival was 100%), FC: feed consumed (g DM), T: time of experiment (84 d), DM: dry matter, AIA: acid insoluble ash, SC: solid waste collected (g/kg feed), DM: dry matter, CP: crude protein, CF: crude fat, CA: crude ash, St: Starch, E: energy in kcal/g DM, t_{US}= sonication time (s), V_{sample} = sample volume (100 mL), TS_{sample} = Total solids in sample, V_{flush}: volume collected during flushing of sieve stack, TSS_{flush}: Total suspended solids in collected flushing volume, cO_2: cumulative oxygen consumption, V_{tot}: total volume of main incubation (2 L); COD added as acetate, tCOD: total COD.

Parameter	Unit	Formula
Fish performance		
Individual fish weight (W_I)	[g]	W_{Tot}/n
Total fish growth (G_{Tot})	[g]	$G_T=W_{Tot,final}-W_{Tot,initial}$
Geometric mean body weight (W_G)	[g]	$W_G= e^{((lnW(I,final)+lnW(I,initial))/2)}$
Feeding rate (FR)	[%/d]	$FR=W_G/(FC * n * T) * 100\%$
Feed conversion ratio (FCR)	[g/g]	$FCR=FC/G_{Tot}$
Specific growth rate (SGR)	[%W/d]	$SGR=((\ln W_{I,final}-\ln W_{I,initial})/T) * 100\%$
Solid waste production, recovery and composition		
Apparent DM digestibility (ADC_{DM})	[%]	$ADC_{DM}=(1-AIA_{feed}/AIA_{feces}) * 100\%$
Solid waste production (FL)	[g/kg feed DM]	$FL = 1000\ g * (100\%-ADC_{DM})/100\%$
Solid waste recovery (RR)	[%]	RR = SC/FL * 100%
Nitrogen-free extracts (NFE)	[g/kg DM]	NFE = DM-CP-CF-CA
Non-starch polysaccharides (NSP)	[g/kg DM]	NSP= NFE-St
COD in feces, based on energy (COD_E)[a]	[g COD/g DM]	$COD_E = E/(3.40\ kcal/g\ O_2)$

continued on following page

Table 3.2 continued

Ultrasound and particle size		
US specific energy (SE)	[J/kg DM]	$SE = 60\ W * t_{US}/(V_{sample} * TS_{sample})$
DM supplied ($DM_{supplied}$)	[g]	$DM_{supplied} = V_{sample} * TS_{sample}$
DM in 1.2-36 µm fraction ($DM_{S1.2}$)	[g]	$DM_{S1.2} = V_{flush} * TSS_{flush}$
DM in <1.2 µm fraction (DM_{diss})	[g]	$DM_{diss} = DM_{supplied} - DM_{>1.2\ µm}$
Relative DM recovery for sieve size X (RSR_{SX})	[%]	$RSR_{SX} = DM_{SX}/DM_{supplied} * 100\%$
Microparticle production (MP)	[g/kg feed DM]	$MP = 100\%\text{-}ADC_{DM} * 1000\ g * (RSR_{S36} + RSR_{S1.2})$
Total COD sample (COD_{sample})	[g]	$COD_{sample} = V_{sample} * TS_{sample} * COD_E$
OUR test and COD degradability		
O_2 consumption after sample addition ($O_{2sample}$)	[mg]	$O_{2sample} = (cO_{2final} - cO_{2initial}) * V_{tot}$
Average endogenous O_2 consumption rate during sample measurement (rO_{2endo})	[mg/min]	$rO_{2endo} = ((cO_{2final/endo1} - cO2_{initial/endo1})/t_{endo1} + (cO_{2final/endo2} - cO_{2initial/endo2})/t_{endo2})/2 * V_{tot}$
O_2 consumption due to sample addition (sO_2)	[mg]	$sO_2 = O_{2sample} - (rO_{2endo} * t_{sample})$
Yield, for amount of $COD_{acetate}$ added (Y)	[mg/mg]	$Y = (1\text{-}sO_2/COD_{acetate})$
rdCOD in sample ($rdCOD_{sample}$)	[mg]	$rdCOD_{sample} = sO_2/(1\text{-}Y)$
Sample degradability($rd_{\%}COD_{sample}$	[%]	$rd_{\%}COD_{sample} = rdCOD_{sample}/tCOD_{sample} * 100\%$

[a] according to Henken et al. (1986)

3.3 Results

3.3.1 Effect of diet composition

The diets successfully generated a contrast in fecal NSP composition, HNSP feces were high in NSP and low in starch when compared with LNSP feces (Table 3.3). HNSP and LNSP feces showed clear differences between cumulative particle size distributions (Figure 3.2), resulting in a significantly higher median particle size in HNSP feces (Table 3.4). Particulate dry matter content (DM > 1.2 µm) was 70.7 ± 7.1% in HNSP feces and 40.5 ± 8.5% in LNSP feces, respectively (p= 0.002, Figure 3.2). Most noteworthy was the fact, that 25% of the dry matter of HNSP feces was present as particulate matter bigger than 200 µm (Figure 3.2). Fecal dry matter retention on a microscreen of 36 µm was approximately 48% for HNSP feces, whereas it was only 10% for LNSP feces (Table 4). When corrected for dry matter digestibility, the production of microparticles (>1.2 µm – 36 µm) per kg of feed was similar between diets (p= 0.913, Figure 3.3). The initial COD degradability was almost 25% in LNSP feces, and significantly higher than in HNSP feces (p= 0.006, Figure 3.4).

Table 3.3: Fecal waste composition as analyzed (mean ± standard deviation, n= 2). DM: dry matter, NFE: Nitrogen-free extracts (calculated), NSP: Non-starch polysaccharides (calculated). Treatments were compared with a t-test, assuming unequal variances.

Parameter	**Unit**	**HNSP**	**LNSP**	***p***
Crude protein	[g/kg DM]	130.0 ± 10.5	188.4 ± 2.2	0.070
Crude fat	[g/kg DM]	25.2 ± 1.5	37.9 ± 4.9	0.147
NFE	[g/kg DM]	590.4 ± 6.5	495.3 ± 16.2	0.047
Starch	[g/kg DM]	14.0 ± 3.1	310.3 ± 33.5	0.049
NSP	[g/kg DM]	576.4 ± 9.6	185.0 ± 49.8	0.049
COD	[g/kg DM]	1054.9 ± 11.2	1027.9 ± 11.6	0.142
Ash	[g/kg DM]	239.4 ± 5.7	263.8 ± 13.7	0.204

Table 3.4: Particle size distribution and particle production in relation to feed in unsonicated feces (mean ± standard deviation, n= 4). Total dry matter (DM) includes the dissolved/suspended dry matter fraction $\leq$ 1.2 µm.

Parameter	**Unit**	**HNSP**	**LNSP**	*p*
Median DM	[µm]	34.4 ± 11.4	1.0 ± 0.1	0.001
Median particles (>1.2 µm)	[µm]	101.1 ± 29.4	24.3± 0.7	0.002
Microparticles (>1.2-36 µm)	[g/kg feed DM]	50.5 ± 9.7	51.4 ± 11.2	0.913
DM recoverable (>36 µm)	[%]	47.9 ± 4.9	9.9 ± 2.0	<0.001
	[g/kg feed DM]	106.1 ± 10.9	16.6 ± 3.3	<0.001

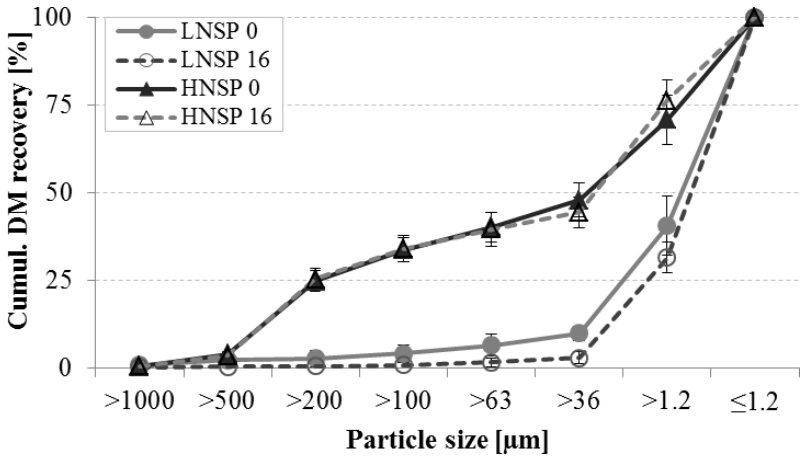

Figure 3.2: Cumulative dry matter recovery for HNSP and LNSP (mean ± standard deviation, n= 4). Only the particle size distribution of the control (t= 0 min) and the maximal sonication time of 16 min is shown. The median particle size corresponds to a cumulative dry matter recovery of 50%.

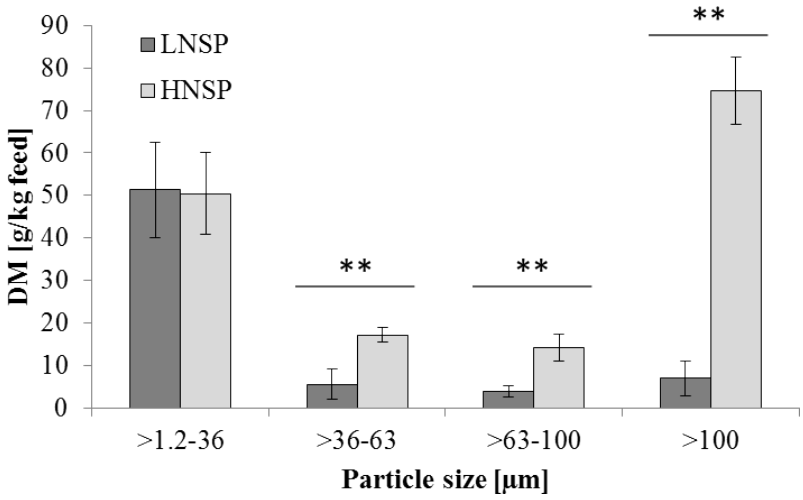

Figure 3.3: Fecal particle generation based on one kg of feed in unsonicated feces (mean ± standard deviation, n= 4). **: significant differences between treatments ($p< 0.01$) Particles between >1.2 µm and 36 µm are considered as microparticles, which could accumulate in RAS. Particles above >36 µm are considered as recoverable. DM: dry matter.

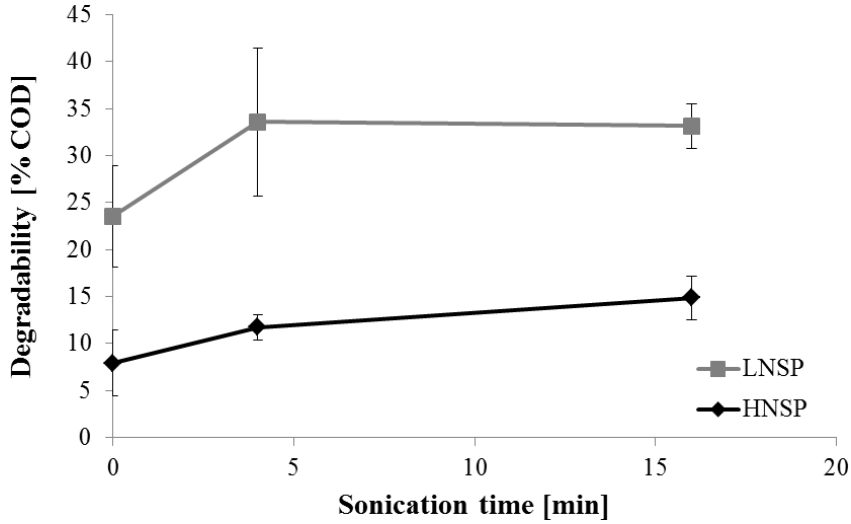

Figure 3.4: COD degradability, determined in oxygen uptake rate test (mean ± standard deviation, n= 4).

3.3.2 Effects of ultrasound treatment

Dry matter contents were significantly different between fecal waste samples (HNSP: 32.6 ± 2.7 g/L; LNSP: 28.5 ± 4.0 g/L; $p< 0.001$), resulting in a ~13% lower specific ultrasound energy (kJ/kg DM) for HNSP when compared to LNSP. The two-way ANOVA showed no effect of sonication time on median particle size (Figure 3.2, $F_{9,4}= 2.157$; $p= 0.098$). However, sonication significantly increased the amount of dry matter $\leq$ 36 µm ($F_{9,4}= 3.688$, $p= 0.015$), showing no interaction effect of diet and sonication time ($F_{9,1}= 0.383$, $p= 0.819$). Sixteen minutes of sonication increased the total amount of dry matter $\leq$ 36 µm by 6.8% and 7.6% for HNSP and LNSP, respectively. Ultrasound treatment had a significant effect on carbon bioavailability in HNSP feces only ($H_2= 6.615$, $p= 0.037$), showing no significant effect in LNSP feces ($H_2= 4.962$, $p= 0.084$). Sixteen minutes of sonication increased COD degradability by 7.0% and 9.6% for HNSP and LNSP, respectively (Figure 3.4).

3.4 Discussion

3.4.1 Feces collection in experimental systems

The increased recovery rate for HNSP feces in sedimentation is consistent with the results of a previous study using the same feeds in RAS (Meriac et al., 2014). However, it is important to acknowledge that the feces used in the sonication experiments represent 68% and 49% of the total feces produced with HNSP and LNSP diets, respectively. Thus, we have to assume that the composition of the collected feces is representative for the total feces produced by the fish. According to Wong and Piedrahita (2000), the upflow velocity of 0.23 cm/s in our settling units should have been sufficient to collect at least 87% of all settleable solids. The collected feces might provide more realistic data when compared to stripped feces, because 100% recovery is not realistic in a practical situation. Nonetheless, further studies could benefit from incorporating a comparison with stripped feces, since our collection method could underestimate the production of small particles and dissolved material.

3.4.2 Effect of diet composition on particle size distribution and carbon bioavailability in feces

The change in diet composition resulted in clear differences in the particle size distribution in HNSP and LNSP feces. HNSP feces consisted of bigger particles and less dissolved material when compared with LNSP feces. After disintegration of the fecal pellet, only 10% of LNSP feces could have been retained on a microscreen of 36 µm, when compared to almost 50% retention for HNSP feces. Comparing size distribution of disintegrated feces to literature values was not possible since we were not aware of other studies investigating particle size distribution in feces with a similar objective and methodology. However, since gelatinized starch has a high digestibility (90%; Hua and Bureau, 2009), the particle size in the feed should decrease during digestion in fish. As a result, the remaining fine-particulate starch could become the pre-dominant microparticle fraction in LNSP feces (Patterson and Watts, 2003a). Accordingly, disintegrated feces from the LNSP diet produced a high amount of fine solids and dissolved material, whereas the HNSP feces showed a prominent fraction of particles between 500 and 200 µm. Lignocellulosic material will pass the fish gut almost unchanged (Lovell, 1998), thus the final particle size distribution in the feces should be a direct result of the grinding size of the ingredients (Besle et al., 1994; Hilton et al., 1983; Patterson and Watts, 2003a). Unfortunately, it was not possible to determine the particle size distribution in the feeds with the same protocol. This was due to a high pellet integrity after starch gelatinization in the extrusion process of the feeds.

The high amount of starch in the LNSP feces, combined with the small particle size, was most likely the main reason for the high short-term degradability of LNSP feces.

Approximately 25% of the COD present in the fecal waste was degraded within 1-2 hours by the bacteria present in the incubation. Klas et al. (2006a) reported a readily degradable COD fraction of 4% in solid waste collected at a fish farm, which corresponds well with the short-term degradability of 8% found in HNSP feces. The short-term degradability of HNSP feces was approximately three times less than LNSP, and substantiates the hypothesis that high NSP contents in feces limit carbon bioavailability. Although the starch content in the LNSP diet of 41% was high when compared to 10-15% starch typically found in practical trout diets, it clearly demonstrates how diet composition can change COD bioavailability in fecal waste.

3.4.3 Effects of ultrasound treatment on fecal waste

Although ultrasound treatment resulted in a small but significant increase of the dry matter fraction below 36 µm, it had no significant effect on the particle size distribution in fecal waste. Effects of ultrasound on particle size and COD degradability are well described in waste water treatment (Pilli et al., 2011), but data on ultrasound treatment of solid waste from fish farming is scarce (McDermott et al., 2001). Many of the studies in waste water treatment determine particle size with non-invasive measurements and observe the breakdown of flocs (Ruiz-Hernando et al., 2010; Tiehm et al., 1997). In contrast, we chose for an invasive method to study the effects of ultrasound on the particles forming the fecal pellet. Thus, the changes in particle size distribution in our experiment were expected to be less dramatic when compared to studies reporting an 85% change of the median (Yagci and Akpinar, 2011). However, the particles in the fecal waste of both diets withstood up to ~20,000 kJ/kg DM without major changes in particle size. Thus, it is unlikely that the particle size will change due to mechanical stress and shearing within RAS after the disintegration of the fecal pellet. This observation could corroborate the hypothesis of McMillan et al. (2003) that the particle size distribution will reach an equilibrium in RAS after the disintegration of solid waste.
Ultrasound treatment increased COD biodegradability by 7-10%. The observed effect is comparable to results of McDermott et al. (2001), who showed that US treatment of aquaculture effluents increased COD removal in a biogas reactor from 77% to 85%. Based on 4 minutes of sonication time, we estimated that approximately 90,000 kJ (25 kWh) are necessary to generate 1 kg of biodegradable COD. A more promising approach to increase carbon bioavailability for denitrification would be the sonication and re-use secondary sludge from the denitrification reactor, which consists to a big part of microbial biomass (Gonze et al., 2003; Mao et al., 2004; Vaxelaire et al., 2008).

3.4.4 Extrapolation for system design

Fecal dry matter recovery on microscreens was significantly increased for the HNSP diet when compared to the LNSP diet. The results suggest that ~48% of the particles

(~34% total DM) originating from fully disintegrated HNSP feces could still be recovered with a microscreen of 100 µm. In contrast, only ~10% of the particles (~4% total DM) of LNSP feces could have been recovered with a screen size of 100 µm. However, our 48% recovery rate for HNSP particles by a 100 µm microscreen illustrates the worst case scenario of total disintegration of the fecal pellet, which should be hardly the case under applied RAS conditions. In comparison, reported drum filter recovery rates of solid waste in commercial RAS range from 30% to 80% for a size range of 40-100 µm (Timmons and Ebeling, 2007). In our experiments, a reduction of microscreen size from 100 µm to 36 µm would improve dry matter recovery at its best by 15% for HNSP feces. Thus, selection of microscreen filters for RAS should go hand in hand with feed formulation to design the most efficient system in the future. If an otherwise stable fecal pellet (produced with an optimized diet) breaks up into easily recoverable particles, smaller drum filters with bigger screen sizes could be used, significantly reducing head loss, investment costs and water exchange at equal system performance. However, due to a lower dry matter digestibility of HNSP diets, the overall load of microparticles onto a system might not be necessarily lower when compared to LNSP diets. If highly digestible and/or micronized ingredients are used for diet formulation, the inclusion of binders should be considered to ensure good system performance.

3.5 Conclusions

The inclusion of unpurified plant ingredients can increase particle size, improve particle recovery with microscreens and hamper COD short-term biodegradability in feces of rainbow trout. Ultrasound treatment had only very limited effects on the particle size distribution in feces, the particles forming the fecal pellet showed to be very resilient to mechanical stress. Although ultrasound treatment increased carbon bioavailability by 7-10%, we conclude that ultrasound is not a feasible conditioning method to increase carbon bioavailability for denitrification in RAS.

3.6 Acknowledgements

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4 Denitrification on internal carbon sources in RAS is limited by fibers in fecal waste of rainbow trout

Submitted to Aquaculture

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Abstract

Denitrification on internal carbon sources offers the advantage to control nitrate levels in recirculating aquaculture systems (RAS) by using the fecal carbon produced within the husbandry system. However, it is not clear to which extend fecal carbon can be utilized by the microbial community within a denitrification reactor. Especially fibers can hamper the bioavailability of carbon in fecal waste. Therefore, this study investigated the nitrogen removal capacity of a denitrification reactor using fecal waste with a high fiber content as the only carbon source in RAS. Furthermore, we investigated to which extent fibers were utilized as a carbon source within the reactor. Four identical small-scale RAS (V= 460 L) were stocked with 25 rainbow trout of ~110 g, and operated at a water exchange rate of ~200 L/kg of feed. Two RAS served as controls without denitrification and two RAS were upgraded with an upflow sludge blanket denitrification reactor (V= 10.5 L). During the six weeks of experiment, we determined COD (chemical oxygen demand, measure for organic carbon) and N balances for all systems and analyzed the composition of the collected solids. The denitrification reactors were able to remove 19 g N/ kg of feed, or 48% of the metabolic nitrogen waste produced by the fish. Based on the COD balances, 44% of the supplied fecal COD was degraded in the reactor. Hemicellulose and cellulose degradability was ~50%, accounting for 45% to the total degraded COD. Under steady state conditions, 4.41 g of biodegradable COD were necessary to remove one gram of nitrogen, indicating respiratory COD losses of approximately 50% in the reactor due to oxygen contamination. This experiment successfully demonstrated that denitrification on internal carbon sources using a high fiber diet could remove half of the nitrogen waste produced by the fish. Although fibers limited carbon bioavailability, half of the cellulose and hemicellulose present in the fecal waste was utilized in the denitrification reactor.

4.1 Introduction

Recirculating aquaculture system (RAS) technology has been successfully adopted for the land-based production of salmon and trout, and the number of farms using RAS keeps on increasing (Martins et al., 2010). However, the use of RAS is often limited by the high energy demand of the systems, and by regulatory limitations on water intake and discharge. To resolve the bottleneck of water intake and discharge, nitrate levels in fish husbandry systems must be controlled. This is usually done by water refreshment, which will eventually determine the minimal water intake and discharge of a husbandry system (Timmons and Ebeling, 2007). Besides controlling nitrate levels in RAS by water refreshment, the accumulation of nitrate can also be counteracted by using denitrification reactors (Balderston and Sieburth, 1976). Denitrification is an anoxic microbial process using organic carbon to convert nitrate into nitrogen gas, generating CO_2 and alkalinity (Equation 4.1). Usually, external carbon sources like methanol or acetate are used to fuel a denitrification reactor (van Rijn et al., 2006). Some systems even use the fecal waste, which is produced within the husbandry system, as the only carbon source for denitrification (Gelfand et al., 2003; Martins et al., 2010). The advantage of using fecal waste as an internal carbon source is the concurrent reduction of the solid waste stream, of which the further treatment and disposal is often a costly process. Thus, denitrification on internal carbon sources looks like an elegant solution to reduce the water demand and waste production in RAS (Martins et al., 2010; van Rijn et al., 2006). However, the efficiency of denitrification on internal carbon sources is usually limited by the amount of carbon available for the microbial community within a denitrification reactor (Klas et al., 2006a). Meriac et al. (2014) showed that the carbon bioavailability in fecal waste of fish was significantly reduced by fibers originating from unpurified plant ingredients in the feed. Fibers can only be used as a carbon source by the bacteria within a denitrification reactor after hydrolysis, which is considered the rate limiting step in the degradation of organic material (Hendriks and Zeeman, 2009; Noike et al., 1985). Therefore, the question arises to which extend fecal waste with a high fiber content can be utilized within a denitrification reactor. This will become a more important issue, considering the trend for an increased substitution of fish meal with plant-based ingredients (Naylor et al., 2009). Therefore, we designed an experiment [i] to investigate the nitrogen removal capacity of a denitrification reactor using fecal waste with a high fiber content as the only carbon source, and [ii] to determine to which extend cellulose, hemicellulose and lignin in fecal waste can be utilized as a carbon source in denitrification.

(4.1) $5\ CH_3OH + 6\ NO_3^- \longrightarrow 3\ N_2 + 5\ CO_2 + 7\ H_2O + 6\ OH^-$
[Denitrification, using methanol as a model substrate (Tchobanoglous et al., 2004).]

4.2 Material and methods

4.2.1 Experimental design

Four identical RAS were used in this experiment, of which two served as control systems and two were upgraded with an upflow sludge blanket denitrification reactor. However, those four systems were part of a larger experiment, which was designed to investigate two independent research questions in a total of six RAS. The two remaining RAS were used to test a different hypothesis, and the obtained results are not included in this paper. The experiment was approved by the Ethics Commission for Animal Experiments of Wageningen University (Reference number 2013016.c).

4.2.2 System design and management

Before the start of the experiment, sodium nitrate was used to set the start concentration of NO_3-N to approximately 120 mg/L in all RAS. The system layout is shown in Figure 4.1. The total system volume of the controls and denitrification set-ups was 460 L and 470 L, respectively. The peristaltic pumps (Masterflex L/S standard digital, using Easy Load II pump heads and A60 F L/S 17 tubing; Metrohm Applikon B.V., Schiedam, The Netherlands) which supplied the denitrification reactors, were connected to an outlet in the feces collection bottles. The bottles were stirred with a magnetic stirrer (~60 rpm) to keep the feces in suspension, so that they could be pumped into the reactor. Feces and system water was pumped continuously into the reactor at ~60 mL/min, the operating conditions of the reactors can be found in Table 4.1. Sludge bed height was recorded daily, the maximum sludge bed height was set to 140 cm (V= 8.9 L). Each reactor was equipped with a stirrer to mix the sludge for degassing and controlling biomass growth on the reactor walls (10 rpm, 30 s on, 30 s off). Fecal COD was used as the only carbon source for the denitrification reactors, and feed spill was avoided at all times. To keep the pH of the experimental systems between 7 and 7.5, sodium bicarbonate was added when necessary. Evaporation losses were compensated in the mornings before the measurements. Water was exchanged before the morning feedings, at a rate of approximately 200 L/kg feed DM. The water was discharged at the sump (Figure 4.1), and the discharge volume was determined indirectly with water meters by measuring the volume necessary to re-fill the systems (0.0001 m^3, Flodis, Schlumberger, Dordrecht, The Netherlands).

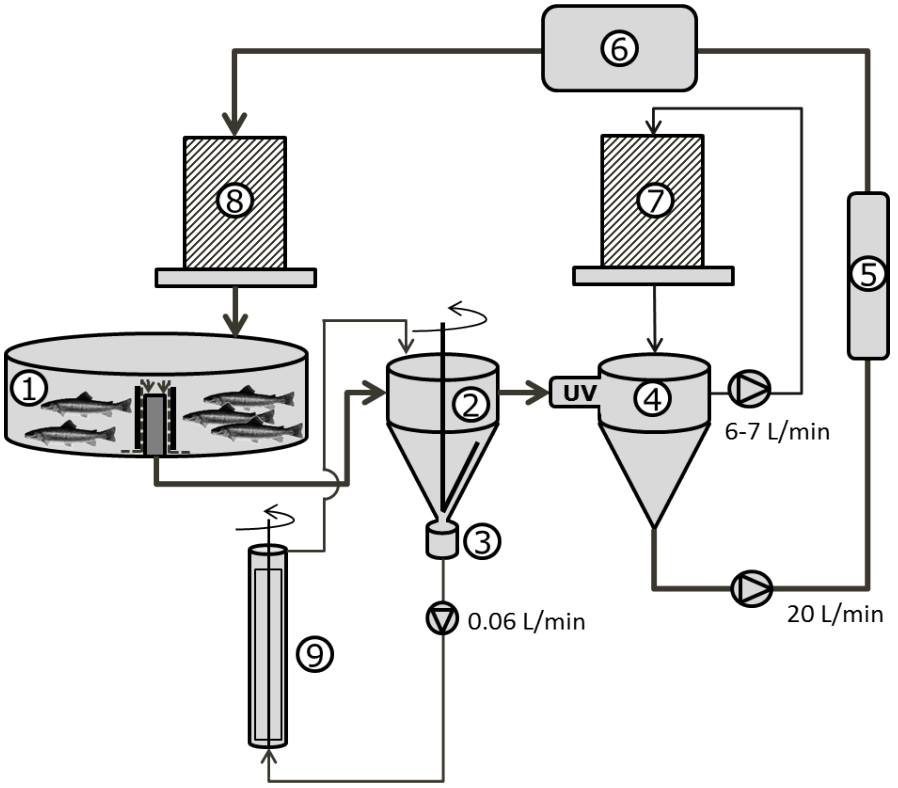

Figure 4.1: RAS layout; adapted from Meriac et al. (2014). [1] fish tank with double stand pipe (V= 300L, A= 0.72 m^2), [2] settling tank w/ stirrer to clean tank walls every 30 min (V=75L, HSL: 150 $m^3/m^2/d$); [3] Collection bottle connected to settling tank, cooled to 4 °C in control systems for feces collection (V= 250 ml); [4] Sump (V= 75L) and UV (UV-C 36 W, Phillips, Eindhoven, The Netherlands), water exchange and sampling point; [5] flow meter; [6] cooler-heater (TC20, Teco, Ravenna, Italy), [7] &[8] trickling filter w/ cross flow medium (V=0.059 m^3, A= 15.8 m^2 each), [9] upflow-sludge blanket denitrification reactor w/ stirrer, connected to solids collection bottle (only in denitrification systems, V_{active}= 10.5 L, HSL: 12.8 $m^3/m^2/d$).

Table 4.1: Denitrification reactor description. HSL: hydraulic surface load, HRT: hydraulic retention time (mean ± standard deviation, n=2).

Parameter	Unit	Value
Active volume	[L]	10.5
Max. sludge bed volume	[L]	8.9
Reactor flow	[mL/min]	56.8 ± 0.8
HSL	[$m^3/m^2/d$]	12.8 ± 0.2
Upflow velocity	[m/h]	0.5 ± 0.0
HRT	[min]	185 ± 2.7

4.2.3 Fish and feeding

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were obtained from Mohnen GmbH (Stolberg, Germany), stocked into flow-through systems at 15 °C and adapted to the experimental diet for four weeks before the start of the experiment. At the start of the experiment, the fish were weighed batch-wise and stocked in the experimental systems. Each system was stocked with 25 fish of ~110 g individual body weight. The photoperiod was set to 12:12 h light/dark with day break at 8:00 h. We used an extruded floating feed with a pellet size of 3 mm (Table 4.2), which is similar to the HNSP diet previously used in Meriac et al. (2014). The fish were fed by hand twice a day at 10:00 h and 16:00 h. Feeding was done at a restricted level of 1.3% body weight/d on average. Feed fines were removed by sieving before feeding. The feed was produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) and contained AIA as an inert marker for digestibility determination (Atkinson et al., 1984; Vandenberg and De La Noue, 2001). Apparent digestibility and fecal recovery was determined in the control systems and assumed to be equal between treatments.

Table 4.2: Feed ingredients and analyzed proximate composition of feed. NFE: nitrogen-free extract (calculated), NSP: non-starch polysaccharides (calculated), FW: fresh weight, DM: dry matter.

Ingredients	**Unit**	**Value**
Fish meal[a]	[g/kg FW]	250
Fish oil[b]	[g/kg FW]	82
Soy bean meal[c]	[g/kg FW]	150
Wheat flour[d]	[g/kg FW]	175
Sunflower seed meal[e]	[g/kg FW]	150
Rape seed meal[f]	[g/kg FW]	150
Monocalciumphosphate[g]	[g/kg FW]	5
L-Lysine HCL[h]	[g/kg FW]	4
DL-Methionine[i]	[g/kg FW]	4
Diamol[j]	[g/kg FW]	20
Premix[k]	[g/kg FW]	10
Proximate composition		
Crude protein	[g/kg DM]	409
Crude fat	[g/kg DM]	129
Crude ash	[g/kg DM]	96
NFE	[g/kg DM]	366
Energy	[g/kg DM]	21
COD	[g/kg DM]	1482
Acid-insoluble ash	[g/kg DM]	17
Starch	[g/kg DM]	150
NSP	[g/kg DM]	215
Cellulose	[g/kg DM]	48
Hemicellulose	[g/kg DM]	57
Lignin	[g/kg DM]	24

[a] TripleNine Fish Protein, Esbjerg, Denmark. [b] Coppens International, Helmond, The Netherlands. [c] Cargill, Amsterdam, The Netherlands. [d] Meneba,Weert, The Netherlands. [e] Arkervaart-Twente, Leusden, The Netherlands. [f] ADM, Spyck, Germany. [g] Tessenderlo Chemie, Rotterdam, The Netherlands. [h] Sewon Paik Kwang Industrial Co. Ltd., Jeollabuk-do, South Korea. [i] Evonik, Hanau, Germany. [j] Damolin A/S, Fur, Denmark. [k] Premix, includes vitamins,minerals and trace elements.

4.2.4 Sampling

At the beginning of the experiment, the fish were anesthetized with 2-phenoxy-1-ethanol (0.1 mL/L), weighed in batches and distributed between the experimental systems. During this procedure, a random sample of 10 fish was taken to determine initial body composition. At the end of the experiment, all fish were weighed in batches, and 10 fish were taken from each tank as a sample to determine final body composition. All sampled fish were euthanized with 2-phenoxy-1-ethanol (1 mL/L), and immediately stored in sealed

plastic bags at -20 °C until the analysis of body composition. The settled fecal waste of the control system was collected twice a day. The collection bottles were disconnected, the supernatant was decanted back into the systems and the fecal waste was then stored in aluminum trays at -20 °C. The supernatant of the solids collection bottles was poured back in to the systems. Excess sludge of the denitrification reactors was collected once a day if necessary. If the maximal sludge bed height of 140 cm was exceeded, the excess sludge was drained using a tap at 140 cm. The drained sludge was collected in aluminum trays and stored at -20 °C. At the last day of the experiment, the sludge was drained from the reactors and allowed to settle. The supernatant was poured back into the systems and the settled sludge was stored in aluminum trays at -20 °C. Feed samples of 100 g were taken every week, and analyzed as a pooled sample at the end of the experiment. Water samples were taken at the start of the experiment, then once weekly and on the final day of the experiment. Samples for TAN and NO_x-N were analyzed immediately. Samples for COD were acidified with H_2SO_4 (pH$\leq$ 2) and analyzed after the end of the experiment (APHA 5220 D, 1998). Furthermore, the reactor performance was measured over a period of 24 h in week five and week six of the experiment. Water samples were taken from the reactor in- and outlet at 9:00, 11:00, 13:00, 15:00, 17:00, 19:00, 23:00, 3:00, 7:00 and 9:00 h. All samples were immediately stored on ice and analyzed for TAN, NO_2-N and NO_3-N after the last sampling. The results of the 24 h sampling were used to determine the nitrogen removal performance of the denitrification reactors, assuming steady state conditions. Denitrification performance was determined as volumetric denitrification rate (reactor volume & sludge bed volume) and specific denitrification rate (based on volatile suspended solids in sludge bed, MLVSS). Sludge bed composition was based on the final sample of the reactor sludge taken at the end of the experiment, and was assumed to be constant for the 24 h measurements of week 5 and week 6.

4.2.5 Analyses

Water quality was measured in the solids collector once a day before the morning feeding. Oxygen, pH, temperature and conductivity was measured with a handheld meter (WTW multi 340i, WTW GmbH, Weilheim, Germany). The weekly water samples were analyzed with an autoanalyzer (SAN Plus, Skalar, Breda, The Netherlands) for total ammonia nitrogen (TAN = NH_3-N + NH_4^+-N), (Skalar protocol number. 155-006 w/r), NO_2-N (Skalar protocol number 467-003), NO_x-N (Skalar protocol number 461-318) and urea-N (Skalar protocol number 612-341). NO_3-N was calculated as NO_x-N - NO_2-N. Total nitrogen (TN) content of the water samples was calculated as TN= TAN + NO_x-N + urea-N, and TN was used in the calculations of the dissolved nitrogen balances of the experimental systems. The acidified water samples for COD were analyzed for total COD (APHA 5220 D, 1998). The frozen fish samples were ground and homogenized in a mincing machine (Model TW-R 70, FEUMA Gastromaschinen GmbH, Gößnitz, Germany). The collected fecal waste, excess sludge and reactor sludge was pre-dried at

70 °C before analyses. For sample preparation, feed and fecal waste was ground with a centrifugal grinding mill (Retsch/Brinkmann ZM 100 /w 1.1 mm sieve, Verder NV, The Netherlands). Proximate composition of fish, feed and feces was determined as dry matter (DM; ISO 6496, 1983), crude ash (ISO 5984, 1978), acid insoluble ash (AIA; ISO 5985, 1981), crude fat (ISO 6492, 1999), crude protein (ISO 5983, 1997, crude protein = Kjeldahl-N × 6.25), energy (ISO 9831,1998), crude fiber (NEN/ISO 6865 using fibercap with pore diameter 23 µm), neutral detergent fiber (NDF; ISO 16472, 2006), acid detergent fiber (ADF; NEN/ISO 13906), acid detergent lignin (ADL; NEN/ISO 13906 using P2 crucibles with pore diameter 40-100 µm) and starch (NEN/ISO 15914). Sugar monomers of hemicellulose and other carbohydrates were determined before and after hydrolysis with hydrochloric acid in feed and feces with HPAE-PAD (High Performance Anion Exchange chromatography with Pulsed Amperometric Detection). Hemicellulose monomer content after acid hydrolysis in solids was calculated as the sum of xylose, mannose, arabinose, galactose, rhamnose and fucose monomers (Saha, 2003). Cellulose content was calculated as ADF-ADL.

4.2.6 Calculation and statistics

The calculations are summarized in Table 4.3. COD and nitrogen balances on fish level were calculated based on digestibility and retention in growth. COD and nitrogen balances on system level were based on the calculated metabolic waste and the measured production of solid and dissolved COD and N, taking accumulation and discharge into account. Statistical analyses were performed with SPSS (IBM SPSS statistics, version 20), treatments were compared with T-tests (assuming unequal variances, n= 2).

Table 4.3: Calculations.

W_{Tot}: total fish biomass per tank (initial or final), n: number of fish in tank (constant over experimental period, 100% survival), FC: feed consumed during experiment (g DM fed per tank, no feed refusal occurred during the experiment), T: experimental days (42 d), SC: solids collected in control systems (g/kg feed DM), DM: dry matter, CP: crude protein, CF: crude fat, CA: crude ash, E: energy in kcal/g DM, AIA: acid-insoluble ash, St: Starch n[$C_6H_{10}O_5$], C: cellulose n[$C_6H_{10}O_5$], HC: hemicellulose, based on monomers, and corrected for hydrolysis (Xy+Ma+Ar+Ga+Rh+Fu)/0.9, L: lignin, based on acid detergent lignin (ADL), Xy: xylose ($C_5H_{10}O_5$), Ma: mannose ($C_6H_{12}O_6$), Ar: arabinose ($C_5H_{10}O_5$), Ga: galactose ($C_6H_{12}O_6$), Rh: rhamnose ($C_6H_{12}O_5$), Fu: fucose ($C_6H_{12}O_5$), X: nutrient, RT: retention, FL: fecal loss, NFL: non-fecal loss, Acc: accumulated in system volume, Col: collected as solid waste, Dis: discharged, UL: unexplained losses, C: concentration (g/L), V: discharged volume (L), wi: sampling day of a given week, wj: sampling day after wi, t: day between wi and wj, Q_{PP}: denitrification reactor influent flow of peristaltic pump (L/min).

Parameter	Unit	Formula
Fish performance		
Individual fish weight (W_I)	[g]	W_{Tot}/n
Total weight gain per tank(G_{Tot})	[g]	$G_T = W_{Tot,final} - W_{Tot,initial}$
Geometric mean body weight (W_G)	[g]	$W_G = e^{((lnW(I,final)+lnW(I,initial))/2)}$
Feeding rate (FR)	[%/d]	$FR = W_G/(FC * n * T) * 100\%$
Feed conversion ratio (FCR)	[g/g]	$FCR = FC/G_{Tot}$
Specific growth rate (SGR)	[%W/d]	$SGR = ((\ln W_{I,final} - \ln W_{I,initial})/T) * 100\%$
Solid waste production, recovery and composition		
Apparent DM digestibility (ADC_{DM})	[%]	$ADC_{DM} = (1 - AIA_{feed}/AIA_{feces}) * 100\%$
Solid waste production (FL)	[g/kg feed DM]	$FL = 1000\ g * (100\% - ADC_{DM})/100\%$
Solids recovery in controls ($RR_{Control}$)	[%]	$RR_{Control} = SC/FL * 100\%$
Nitrogen-free extract (NFE)	[g/kg DM]	NFE= DM-CP-CF-CA
Non-starch polysaccharides (NSP)	[g/kg DM]	NSP= NFE-St
Remainder (R)	[g/kg DM]	R = NSP – C – HC – L
COD in solids, based on energy (COD_E)[a]	[g COD/g DM]	$COD_E = E/(3.40\ kcal/g\ O_2)$
COD crude protein[a]	[g COD/g DM]	$COD_{CP} = 1.66\ g\ O_2/g\ CP$
COD crude fat[a]	[g COD/g DM]	$COD_{CF} = 2.78\ g\ O_2/g\ CF$
COD starch (COD_{St})[b]	[g COD/g DM]	$COD_{St} = 1.19\ g\ O_2/g\ St$
COD cellulose (COD_C)[b]	[g COD/g DM]	$COD_C = 1.19\ g\ O_2/g\ C$
COD hemicellulose (COD_{HC})[b]	[g COD/g DM]	COD_{HC}= (Xy+Ma+Ar+Ga)*1.07 g O_2/g+ (Rh+Fu)*1.27 g O_2/g
COD remainder (COD_R)[c]	[g COD/g DM]	$COD_R = R * 1.19\ g\ O_2/\ g\ R$
COD lignin (COD_L)[d]	[g COD/g DM]	$COD_L = COD_E - COD_{CP} - COD_{CF} - COD_{St} - COD_C - COD_{HC} - COD_R$

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Table 4.3 continued

Nutrient balances (COD and N)		
Nutrient (X) mass balance on fish level	[g]	$X_{FC} = X_{RT} + X_{FL} + X_{NFL}$
Nutrient (X) mass balance on system level (X= COD or N)	[g]	$X_{FC} = X_{RT} + X_{Acc} + X_{Col} + X_{Dis} + X_{UL}$ $X_{Acc} = X_{sys\_ final} - X_{sys\_ initial}$ $X_{Dis} = \sum_{t=1}^{42d}(C_{Xt} * V_t)$
Nutrient concentration between sampling days (C_{Xt})	[g/L]	$C_{Xt} = (C_{wj}-C_{wi})/(t_{wj}-t_{wi}) * (t-t_{wi}) + C_{Xwi}$
Suspended/diss. nutrient production in system (X_{Pro})	[g]	$X_{Pro} = X_{Acc} + X_{Dis}$
Susp./dissolved nutrient production in system (X_{Pro})	[g/kg feed]	$X_{PR} = X_{Pro}/(FC/1000)$
Passive denitrification in control systems (DN_{Pass})	[g/kg feed DM]	$DN_{Pass} = N_{UL,control}/(FC/1000)$
Nitrogen removal due to denitrification (DN_{Act})	[g/kg feed DM]	$DN_{Act} = N_{UL,denitrification}/(FC/1000) - DN_{Pass,\ control}$
Nitrogen reactor load (N_{RLD})	[g.kg feed DM]	$N_{RLD} = (N_{NFL} + N_{FL}/(FC/1000)$
COD reactor load (COD_{RLD})	[g/kg feed DM]	$COD_{RLD} = (COD_{FL} * RR_{Control})/(FC/1000)$
COD in residual solids (COD_{RS})	[g/kg feed DM]	$COD_{RS} = (COD_{Reactor\ sludge} + COD_{Excess\ sludge})/(FC/1000)$
COD degraded in reactor (COD_{DR})	[g/kg feed DM]	$COD_{DR} = COD_{RLD} - COD_{RS}$
COD degradability (COD_{Deg})	[%]	$COD_{Deg} = COD_{DR}/COD_{RLD} * 100\ \%$
Reactor performance (24 h measurements)		
Total nitrogen (TN) concentration change (ΔC_t)	[mg/L]	$\Delta C_t = TN_{in,t}-TN_{out,t}$
TN removal between samplings (ΔN_{ti-tj})e	[mg]	$(\Delta N_{ti-tj} = (\Delta C_{tj} + \Delta C_{ti})/2 * Q_{PP} * t_{(j-i)}$
TN accumulation in reactor (N_{Racc})[e]	[mg]	$N_{Racc,\ ti-tj} = ((TN_{in,tj} + TN_{in,ti})/2 - (TN_{out,tj} + TN_{out,ti})/2) * V_{reactor}$
TN removal between two measurements (N_{NR})[e]	[g]	$N_{NR,ti-tj} = (\Delta N_{ti-tj} + N_{Racc,\ ti-tj})/1000$
TN removal during 24 h (N_{24h})	[g]	$N_{24h} = \sum_{n=1}^{10} N_{NR}$ (10 measurements during 24 h)
TN removal per kg of feed (N_{DNR})	[g/kg feed DM]	$N_{DNR} = N_{24h}/0.048$ kg feed DM (constant during week 5 & 6)
Volumetric denitrification rate (DN_{RV})	[mg/L/d]	$DN_{RV} = N_{24h}/V_{Sludge\ bed} * 1000$
Specific denitrification rate (DNR_S)[f]	[g/kg MLVSS/d]	$DNR_S = N_{24h}/((DM_{Reactor\ sludge} - CA_{Reactor\ sludge})/1000)$
Excess sludge production (ESP)[g]	[L/kg feed DM]	$ESP = V_{Excess\ sludge}/(FC/1000)$
COD/N ratios		
COD/N in metabolic waste (COD/N_{MW})	[g/g]	$COD/N_{MW} = COD_{FL}/(N_{NFL}+N_{FL})$
COD/N reactor load (COD/N_{RL})	[g/g]	$COD/N_{RLD} = COD_{RLD}/N_{RLD}$
COD/N requirement for N removal (COD/N_{NR})	[g/g]	$COD/N_{NR} = COD_{DR}/DN_{Act}$

[a]Based on Henken et al. (1986), [b]Stoichiometric oxygen demand calculated acc. to Tchobanoglous et al. (2004), [c]Assumed to be identical with other carbohydrates as starch and cellulose,[d]We determined an average COD/lignin ratio of 1.76 g/g in feces and residual solids. This seems comparable to estimated COD ratios of 1.85 – 1.87 g/g, derived from Zhang et al. (2012)(lignin from prairie cord grass & aspen, no universal structural formula for lignin could be found in literature), [e]Adapted from Saravanan et al. (2012), [f] Sludge composition is based on the residual solids remaining in the reactor at the end of the experiment, [g]Excess sludge production is based on the discharged/collected sludge when maximal sludge bed height was exceeded in the reactor.

4.3 Results

4.3.1 Water quality, fish performance and system management

NO_2-N and NO_3-N concentration were significantly lower in RAS with denitrification when compared to the control systems ($p< 0.05$, Table 4.4). At the end of the experiment, the average NO_3-N levels in denitrification systems were 100 mg/L against 160 mg/L in the control systems (Figure 4.2). No significant differences in fish performance were observed between treatments (Table 4.5). Systems with denitrification consumed only 87 g of sodium bicarbonate when compared to 211 g in the controls. Analysis of the fecal waste collected in the control systems showed that apparent digestibility and fecal recovery was comparable to results previously obtained by Meriac et al. (2014)(Table 4.6). During data analysis, we discovered that the water exchange rate was systematically overestimated in one of the control systems, resulting in a lower actual water exchange for this system (RAS6). The measured average evaporation in RAS6 was 1.1 L/d whereas it was on average 3.7 ± 0.09 L/d in the rest of the systems (n=3). This water debt of 2.6 L was compensated during water exchange, thus systematically overestimating the true refill volume after discharge. The water balance for RAS6 needed to be corrected for the calculated water debt, resulting in an actual water exchange rate of 147.4 L/kg feed.

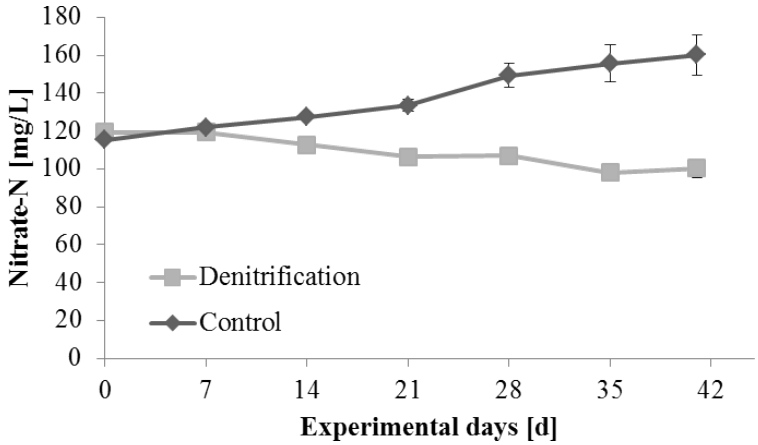

Figure 4.2: Nitrate-nitrogen concentration in the systems (mean ± standard deviation, n=2).

Table 4.4: Water quality and system management (mean ± standard deviation, n=2).

		Systems		
Parameter	**Unit**	**Control**	**Denitrification**	***p***
T	[°C]	15.3 ± 0.17	15.3 ± 0.24	0.880
pH		7.3 ± 0.12	7.3 ± 0.04	0.881
O_2	[mg/L]	9.0 ± 0.01	9.0 ± 0.03	0.095
Conductivity	[mS/cm]	2.7 ± 0.11	2.4 ± 0.04	0.110
TAN	[mg/L]	0.3 ± 0.16	0.1 ± 0.03	0.327
NO_2-N	[mg/L]	0.5 ± 0.05	0.2 ± 0.07	0.048
NO_3-N	[mg/L]	137.6 ± 4.27	108.9 ± 1.88	0.013
tCOD	[mg/L]	36.9 ± 4.33	26.4 ± 6.35	0.193
Bicarbonate	[g/kg feed DM]	211.2 ± 9.60	86.8 ± 8.68	0.005
Water exchange	[L/kg feed DM]	169.9 ± 31.91	194.8 ± 1.48	0.385

Table 4.5: Fish performance and feeding (mean ± standard deviation, n=2). W_G: geometric mean body weight, FCR: Feed conversion ratio, SGR: specific growth rate.

		Systems		
Parameter	**Unit**	**Control**	**Denitrification**	***p***
Initial body weight	[g]	113.6 ± 2.6	112.3 ± 1.1	0.606
Final body weight	[g]	180.9 ± 2.0	182.0 ± 2.4	0.673
Survival	[%]	100 ± n.a.	100 ± n.a.	n.a.
Feeding rate	[%/W_G/d]	1.27 ± 0.0	1.28 ± 0.01	0.875
FCR	[kg FW/kg DM]	1.14 ± 0.01	1.10 ± 0.02	0.198
SGR	[%/d]	1.11 ± 0.03	1.15 ± 0.01	0.250

Table 4.6: Apparent digestibility of feed, as determined in the control systems (mean ± standard deviation, n=2). NFE: nitrogen-free extract (calculated), NSP: non-starch polysaccharides (calculated).

Parameter	Unit	Value
Dry matter	[%]	76.2 ± 0.24
Crude protein	[%]	92.6 ± 0.20
Crude fat	[%]	95.5 ± 0.28
Crude ash	[%]	39.9 ± 0.36
NFE	[%]	60.4 ± 0.23
COD	[%]	83.1 ± 0.32
Starch	[%]	96.3 ± 0.01
NSP	[%]	35.5 ± 0.39
Cellulose	[%]	-2.1 ± 6.39
Hemicellulose	[%]	39.9 ± 2.45
Lignin	[%]	-10.1 ± 4.08
Recovery[a]	[%]	81.3 ± 1.29

[a] Expressed as % undigested dry matter recovered.

4.3.2 Nitrogen production and reactor performance

Nitrogen retention was similar between treatments, branchial and urinary nitrogen (BUN) losses of the fish were ~35 g N/ kg feed dry matter (p= 0.949, Table 4.7). Passive denitrification, which was determined in the control systems, removed 1.2 g N/kg feed DM. After correcting for passive denitrification, the denitrification reactors removed 19 g of nitrogen per kg of feed DM over the whole experimental period. It took the denitrification reactors two to three weeks to start-up, and to stabilize the nitrogen removal rate (Figure 4.3). The 24 hour measurements of the reactor performance in week five and six showed that nitrogen removal under steady state conditions was 20.6 ± 1.2 g N/kg of feed DM (Figure 4.4). The results were pooled, since no significant differences were found between reactors or weeks (p> 0.05). The resulting volumetric nitrogen removal rate in the reactors was 94.7 mg N/L/d, or 111.7 mg N/d per liter of sludge bed, and 8.6 g N/d per kg of organic matter of sludge (Table 4.8).

Table 4.7: Nitrogen balance over the whole experimental period, expressed as g N per kg of feed DM (mean ± standard deviation, n=2). BUN: branchial and urinary nitrogen losses.

	Systems		
Parameter	**Control**	**Denitrification**	***p***
Feed input	65.5 ± n.a.	65.5 ± n.a.	-
Fish retention	25.8 ± 0.8	25.7 ± 0.4	0.941
Metabolic waste	39.7 ± 0.8	39.8 ± 0.4	0.941
... as BUN	34.8 ± 0.9	34.9 ± 0.4	0.949
... as fecal waste[a]	4.9 ± 0.1	4.9 ± n.a.	-
Total N production	38.5 ± 0.9	19.6 ± 2.1	0.025
... as dissolved N	34.5 ± 0.9	16.7 ± 1.9	0.019
... as solid N	4.0 ± 0.0	2.8 ± 0.2	0.033
Total N removed	1.2 ± 0.1	20.2 ± 1.7	0.040
N removed in active denitrification	0	19.0 ± 1.7	-

[a] Determined in control systems.

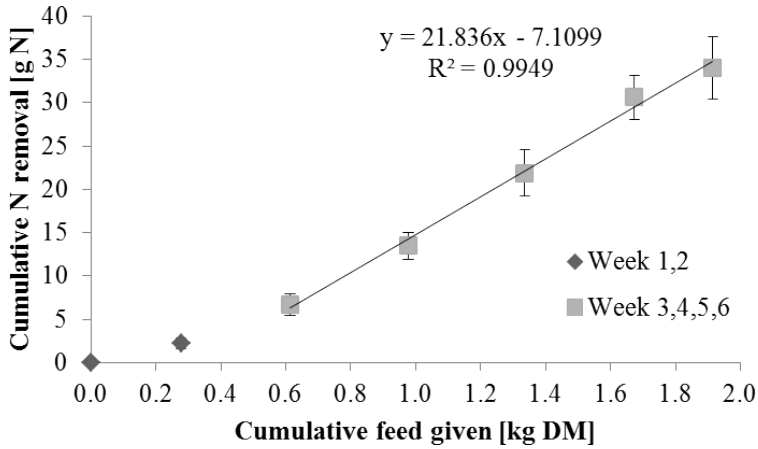

Figure 4.3: Cumulative nitrogen removal per kg of feed given (mean ± standard deviation, n=2). The nitrogen removal rate of the reactors stabilized after 2 weeks of experiment.

Table 4.8: Reactor performance (mean ± standard deviation). Denitrification rates were calculated based on the nitrogen removal during the 24 h measurements in week 5 and 6 (n= 4). The results were pooled, since no significant difference were observed between weeks or reactors (p> 0.05). Sludge retention time was based on excess sludge production after the final sludge bed height was reached (n= 2). MLVSS: mixed-liquor volatile suspended solids.

Parameter	Unit	Value
Volumetric denitrification rate	[mg/L reactor/d]	94.7 ± 5.7
	[mg/L sludge/d]	111.7 ± 6.7
Specific denitrification rate	[g/kg MLVSS/d]	8.6 ± 0.4
Excess sludge production	[L/kg feed DM]	21.7 ± 1.2
Sludge retention time	[d]	9.0 ± 0.5

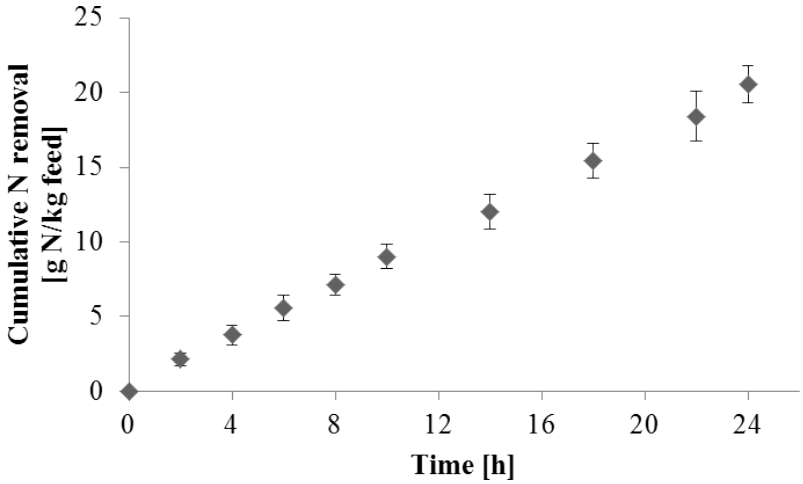

Figure 4.4: Nitrogen removal during 24 h-measurements in week 5 and 6 (mean ± standard deviation, n= 4). The starting time of the measurements was 9:00 of the respective sampling day (t=0). No significant differences in cumulative N removal were observed between weeks or reactors and the results were pooled ($p > 0.05$).

4.3.3 COD balances and COD/N ratios

Based on digestibility and recovery in the control systems, 204 g of COD were supplied to the denitrification reactors per kg of feed DM (Table 4.9). Excess sludge and reactor residue accounted for 113 g of the supplied COD, and 90 g of COD were removed in the denitrification reactors. Dissolved/suspended COD production was 9.0 ± 2.0 g/kg and 10.3 ± 2.4 g/kg feed DM for denitrification systems and controls, respectively, showing no significant difference between treatments (p= 0.612). Based on the COD balances of the reactors, 44.4 ± 1.9 % of the supplied COD was degraded. The reactors were operated at a COD/N ratio of 5.3 (Table 4.10). Under steady state conditions, 4.4 g of biodegradable COD were necessary to remove one gram of nitrogen in the reactors. Full denitrification could be realized, if the reactor was supplied with a COD/N ratio of 9.9.

Table 4.9: COD balance for denitrification systems, as g COD per kg of feed DM (mean ± standard deviation, n=2). Respiratory COD losses in fish are calculated based on the assumption that all non-fecal COD is respired ($COD_{respiration} = COD_{feed} - COD_{retention} - COD_{fecal\ loss}$, of which $COD_{fecal\ loss}$ was determined in the control systems)

Parameter	Unit	Value
Feed input	[g/kg feed DM]	1482 ± n.a.
Fish retention	[g/kg feed DM]	512.9 ± 20.7
Fish respiration	[g/kg feed DM]	718.1 ± 20.7
Fecal waste production[a]	[g/kg feed DM]	250.5 ± 4.7
Dissolved/suspended COD	[g/kg feed DM]	9.0 ± 2.0
System loss[b]	[g/kg feed DM]	37.9 ± 2.0
Supplied to reactor[a]	[g/kg feed DM]	203.7 ± 0.6
Total residual solids	[g/kg feed DM]	113.3 ± 3.8
... as excess sludge	[g/kg feed DM]	24.8 ± 1.7
...as reactor residue	[g/kg feed DM]	88.5 ± 5.5
COD consumed in reactor	[g/kg feed DM]	90.4 ± 3.8

[a] Based on digestibility, determined in the control systems; [b] Calculated as fecal waste – suspended/dissolved COD production – COD supplied to reactor, accounting for COD losses in RAS.

Table 4.10: COD/N ratios in denitrification systems (mean ± standard deviation). N refers to both solid and dissolved nitrogen.

Parameter	Unit	Value
Feed	[g COD/g N]	22.6 ± n.a.
Metabolic waste[a]	[g COD/g N]	6.3 ± 0.1
Reactor load[a]	[g COD/g N]	5.3 ± 0.1
Nitrogen removal ratio[b]	[g COD/g N]	4.4 ± 0.4
COD/N for full denitrification[b]	[g COD/g N]	9.9 ± 0.6

[a] n= 2, [b] Based on the bioavailable COD supplied to the denitrification reactor at steady-state conditions (n=4).

4.3.4 Feces degradability in denitrification reactor

The analysis of fecal waste and reactor residue revealed that cellulose and hemicellulose had a combined degradability of 41.3 ± 1.2%, and made up approximately 45% of the total degraded COD in the reactor (Figure 4.5). The results indicate that lignin was not degraded in the reactor at all (Table 4.11). The remaining dry matter consisted of approximately 50% hemicellulose, cellulose and lignin and 28% ash. Although starch had a rather small contribution to the total degraded COD, the degradability of starch in the reactor was very high.

Table 4.11: Dry matter degradability of fecal waste in denitrification reactor (mean ± standard deviation, n= 2).

Parameter	Unit	Value
Dry matter	[%]	44.8 ± 2.2
Crude protein	[%]	29.0 ± 5.2
Crude fat	[%]	62.0 ± 1.6
Crude ash	[%]	36.3 ± 0.8
COD	[%]	44.4 ± 1.9
Starch	[%]	96.4 ± 1.3
Cellulose	[%]	48.7 ± 2.8
Hemicellulose	[%]	60.3 ± 0.1
Lignin	[%]	-3.3 ± 2.0
Remainder	[%]	84.1 ± 13.6

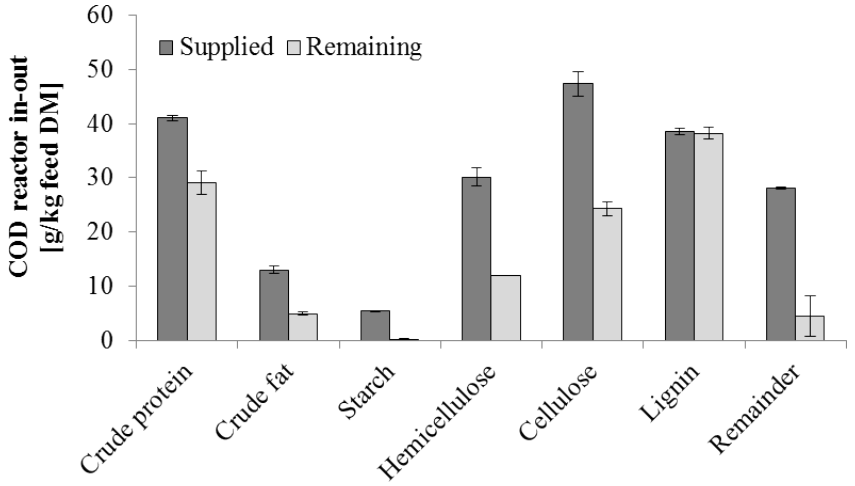

Figure 4.5: COD composition of supplied COD and remaining COD after degradation in the reactor (mean ± standard deviation, n= 2). The COD of the remainder was estimated to be the same as for starch and cellulose (1.19 g COD/g DM). Lignin was calculated as COD_{lignin}= tCOD-$COD_{protein}$-COD_{fat}-$COD_{cellulose}$-$COD_{hemicellulose}$-$COD_{remainder}$.

4.4 Discussion

4.4.1 Water quality and management

As expected, the denitrification reactors were able to control nitrate levels in the systems. Although literature suggests that incomplete denitrification can result in the additional production of nitrite (Balderston and Sieburth, 1976), we found lower nitrite concentrations in denitrification systems when compared to the control systems. A possible explanation could be that the additional alkalinity from denitrification systems compensated a lack of alkalinity in RAS controls without denitrification (van Rijn et al., 2006). Accordingly, the demand of bicarbonate to stabilize pH in denitrification systems was reduced by ~60% when compared with the control systems. Despite the differences in nitrite concentrations between treatments, no significant effects on fish performance were observed.

4.4.2 COD and N balances

Due to a lower feeding rate and lower growth, the nitrogen retention in fish was only 39% when compared to 48% obtained in the previous study of Meriac et al. (2014). This resulted in a higher nitrogen production rate of ~35 g N/kg feed (BUN), when compared to 29 g N/kg feed in the previous study. Systems with denitrification reactors removed approximately 50% of the solid and dissolved nitrogen waste produced by the fish, using

fecal COD as the only carbon source. Only 3% of the nitrogen losses could be accredited to passive denitrification in the experimental systems. The specific nitrogen removal rate of the reactor was 8.6 g N/kg MLVSS/d, which is lower compared to an estimated specific removal rate of 21-25 g N/kg MLVSS/d of Klas et al. (2006b)(SRT ~11d). However, this difference could probably be explained by a lower temperature in our denitrification systems compared to Klas et al. (2006b)(15 °C vs. 25 °C).
Due to the high fecal recovery rate and low digestibility of the feed, approximately 200 g of fecal COD per kg of feed were supplied to the denitrification reactors. However, only 80 g of COD per kg of feed was respired by the bacterial community in the denitrification reactor. Remarkably, the determined COD degradability of 44% reproduced successfully the ~44% degradability obtained in a BOD_{10} test of Meriac et al. (2014). This result indicates that the oxygen consumption observed in a BOD_{10} test could be used as a good proxy to estimate the denitrification potential of fecal waste samples under anaerobic conditions.
Because of a lower nitrogen retention in fish, COD/N ratios in the metabolic waste were lower when compared to the data previously obtained by Meriac et al. (2014). The reactors were supplied with a COD/N ratio of 5.3 g COD/ g N. Full denitrification could have been only realized, if the reactor would have been operated at a COD/N ratio of 9.9 g COD/g N. Nitrate removal in the reactors required 4.41 of g biodegradable COD per gram of nitrate, which is ~54% higher than the theoretical COD/N ratio of 2.86 for nitrogen removal (Tchobanoglous et al., 2004). This indicates that more than half of the bioavailable COD was not used for denitrification, but probably respired by the bacteria using the residual oxygen in the water supplying the reactors. Klas et al. (2006b) reported a similar minimal biodegradable COD/N ratio of 4.5 for nitrogen removal in their lab scale denitrification system. Oxidative losses of biodegradable COD remain an important issue and need to be minimized to ensure an optimal utilization of available carbon and maximal reactor performance. A smaller flow across the reactor could minimize the problem of oxygen contamination in the reactor, providing that sufficient nitrate remains in the reactor to avoid the generation of unwanted by-products like sulphides.

4.4.3 Feces degradability in the denitrification reactors

Hemicellulose, cellulose and lignin make up 66% of the COD in the residual solids after digestion. This finding seems to confirm the predictions of van Rijn et al. (1995), who suggested that the undegradable carbohydrate fraction remaining after the anaerobic digestion of fish feed is composed of cellulose and lignin. However, hemicellulose and cellulose seem to be fairly degradable in the denitrification reactor and contribute almost half to the degraded COD. The COD remaining after the degradation of fecal waste still contained 32% hemicellulose and cellulose. This raises the question whether the remaining hemicellulose and cellulose could be degraded further at a longer sludge retention time. If hemicellulose and cellulose was fully utilized in the reactor, the reactor

could potentially remove 90% of the produced nitrogen at the observed COD/N ratio of 4.4. To overcome a limited carbon bioavailability, the use of exogenous enzymes like cellulases and hemicellulases has been discussed (Pérez et al., 2002; Sun and Cheng, 2002). Although lignin is virtually undegradable (Pérez et al., 2002), we were surprised about the high recovery of the lignin in the system. Based on the amount of lignin supplied with feed, approximately 93% was recovered in the remaining solids after digestion of feces in the reactor. Tacon and Rodrigues (1984) suggested the use of crude fiber as an indicator for digestibility studies in fish, the same concept could be applied to lignin. Lignin could be used as a natural marker for in-situ studies on feed digestibility and waste treatment processes in RAS, without the need for special feeds which include an external marker like AIA or yttrium oxide.

4.5 Conclusions

We demonstrated successfully that an upflow-sludge blanket denitrification reactor was able to remove half of the nitrogen waste produced in RAS, by using fecal waste produced in the system as the only carbon source. Although fibers limited the bioavailability of carbon in the fecal waste, approximately half of the COD present as hemicellulose and cellulose in feces was utilized by the reactor. This study shows, that diet composition plays an integral role for the carbon budget for a denitrification reactor.

4.6 Acknowledgements

We would like to acknowledge Interreg IV A and Agentschap NL for funding and all contributors of the EM-MARES and AquaVlan project consortia.

5 Using Viscozyme® L to improve fiber degradability in a denitrification reactor in RAS

In preparation

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Abstract

Denitrification on internal carbon sources can be used to control nitrate and reduce solid and dissolved waste emissions in RAS. The applicability of denitrification on internal carbon sources can be hampered due to the limited carbon availability of the fecal fiber fraction. Enzymatic conditioning has been applied in other biotechnological processes to improve the carbon bioavailability in fibers. The goal of this study was to determine whether enzymatic pre-digestion can improve the degradability of fibers in a denitrification reactor in RAS. Firstly, we determined the optimal hydrolysis conditions in-vitro for fecal waste, which was collected in flow-through systems stocked with rainbow trout. After determining the optimal hydrolysis conditions, we performed a benchmark incubation to determine the amount of sugar liberation under applied conditions. Secondly, we compared fiber degradability of untreated and enzyme-treated fecal waste in denitrification reactors in RAS stocked with rainbow trout. The in-vitro results suggest that approximately 22% of the fecal carbohydrate fraction was converted to reducing sugars, using an enzyme loading of 2.1% (T= 50 °C, pH 4.4, t= 24 h). The analysis of the supernatant of the benchmark incubation revealed that most of the monomers were products of hemicellulose hydrolysis. The degradability of the fecal carbohydrate fraction in the reactors was approximately 50%, showing no significant differences between untreated and enzyme-treated feces. Since no additive effects of the enzymatic pre-digestion were observed, we assume that the fiber fraction remaining as residual solids is especially resilient to degradation. Therefore, these residual solids would be an ideal starting point to screen for more suitable enzymes to increase carbon bioavailability for denitrification in the future. Enzymatic pre-digestion of fecal waste should be able to hydrolyze more than 50% of the fecal carbon fraction to effectively increase carbon bioavailability for denitrification.

5.1 Introduction

Denitrification has been successfully applied in recirculating aquaculture systems (RAS) to control the accumulation of nitrate (Balderston and Sieburth, 1976; Sauthier et al., 1998). Consequently, denitrification can significantly reduce the water demand in RAS (Martins et al., 2009; Timmons and Ebeling, 2007). Denitrification usually requires the addition of external carbon sources like methanol (van Rijn et al., 2006). However, instead of using external carbon sources, several authors successfully used the fecal waste produced within the husbandry system as an internal carbon source for denitrification (Gelfand et al., 2003; Martins et al., 2009; Tal et al., 2009). Using internal carbon sources has the advantages that the costs for external carbon sources are reduced or even eliminated, and that solid waste production of the systems is reduced as well. However, one of the main drawbacks of using internal carbon sources is that fecal waste is not standardized and homogenous, in contrast to carbon sources such as methanol. Depending on fish feed composition, the bioavailability of the fecal carbon can vary considerably between different diets (Chapter 2)(Meriac et al., 2014). A major limiting factor for the carbon bioavailability in feces is the content of fibers, such as cellulose, hemicellulose and lignin. In Chapter 4, we have shown that approximately 50% of the fibers remains undigested in a denitrification reactor at a sludge retention time of 9 days. In biofuel production, enzymes as cellulases and hemicellulases are used to increase the carbon bioavailability of fibers (Pérez et al., 2002). The question arises whether the same approach can be used to increase the bioavailability of the fecal fiber fraction for denitrification. Therefore, this study investigated whether enzymatic pre-digestion can increase the degradability of fibers in the fecal waste in a denitrification reactor in RAS.

5.2 Material and Methods

During the first phase of this study, we focused on optimizing the conditions for enzymatic hydrolysis of dried fecal waste in-vitro. In the second phase, we investigated whether the pre-digestion of fecal waste with enzymes can increase the degradability of fibers in a denitrification reactor in RAS.

5.2.1 In-vitro assays

Feces production and collection for enzyme assays

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were obtained at Mohnen GmbH (Stohlberg, Germany) and kept in two flow-through tanks for the production of fecal waste. Fecal waste for the in-vitro assays was produced using a high fiber diet, the feed ingredient composition (Table 5.1) is identical to the HNSP diet described in Meriac et al. (2014). The fish were fed restrictively at 1.1% body weight per day, the daily ration was

equally divided over the morning and afternoon feeding (9:30 and 16:30 h, respectively). Feed spill was avoided at all times to prevent contamination of the feces collection. The initial individual weight of the fish was 250 g, and the fish grew to a final individual body weight of 503 g. The feed conversion ratio was 1.05 g/g and the specific growth rate was 1.15 %/d.

The flow-through tanks were identical to the experimental set-up described in Chapter 3. Each tank had a volume of ~130 L and the bottom drains were connected to a hydrocylone for feces collection (V= 17 L, Aquaoptima AS, Trondheim Norway). The average water temperature was 15.5 ± 0.2 °C, and the average oxygen concentration in the outlets of the tanks was 6.9 ± 0.8 mg/L. The photoperiod was set to 12:12 light/dark. The flow rate was ~6 L/min per tank, resulting in a hydraulic surface load of 180 m^3/m^2 in the feces collectors. Feces were collected in ice-cooled glass bottles connected to the bottom of the hydrocyclones, similar as described by Saravanan et al. (2012). Twice a day, just before feeding, the feces collection bottles were disconnected from the hydroyclones, the supernatant in the collection bottles was decanted and the feces were stored in aluminum trays at -20 °C. The trays with the collected fecal waste were pre-dried in an oven at 70 °C for 4-6 days. The dried feces were pooled per tank and ground (Retsch/Brinkman ZM 100, 1 mm sieve, Verder NV, The Netherlands). Feed and feces were analyzed for dry matter, crude protein, crude ash and nitrogen-free extract as described in (Meriac et al., 2014)(Chapter 2).

Table 5.1: Feed ingredients and analyzed proximate composition of feed. NFE: nitrogen-free extract (calculated), NSP: non-starch polysaccharides (calculated), FW: fresh weight, DM: dry matter.

Ingredients		
Fish meal[a]	[g/kg FW]	250
Fish oil[b]	[g/kg FW]	82
Soy bean meal[c]	[g/kg FW]	150
Wheat flour[d]	[g/kg FW]	175
Sunflower seed meal[e]	[g/kg FW]	150
Rape seed meal[f]	[g/kg FW]	150
Monocalciumphosphate[g]	[g/kg FW]	5
L-Lysine HCL[h]	[g/kg FW]	4
DL-Methionine[i]	[g/kg FW]	4
Diamol[j]	[g/kg FW]	20
Premix[k]	[g/kg FW]	10
Proximate composition		
Crude protein	[g/kg DM]	409
Crude fat	[g/kg DM]	129
Crude ash	[g/kg DM]	96
NFE	[g/kg DM]	366
Energy	[g/kg DM]	21
COD	[g/kg DM]	1482
Acid-insoluble ash	[g/kg DM]	17
Starch	[g/kg DM]	150
NSP	[g/kg DM]	215
Cellulose	[g/kg DM]	48
Hemicellulose	[g/kg DM]	57
Lignin	[g/kg DM]	24

[a] TripleNine Fish Protein, Esbjerg, Denmark. [b] Coppens International, Helmond, The Netherlands. [c] Cargill, Amsterdam, The Netherlands. [d] Meneba,Weert, The Netherlands. [e] Arkervaart-Twente, Leusden, The Netherlands. [f] ADM, Spyck, Germany. [g] Tessenderlo Chemie, Rotterdam, The Netherlands. [h] Sewon Paik Kwang Industrial Co. Ltd., Jeollabuk-do, South Korea. [i] Evonik, Hanau, Germany. [j] Damolin A/S, Fur, Denmark. [k] Premix, includes vitamins,minerals and trace elements.

Enzyme assays

Viscozyme® L (Lot-No.: SLBB2075V, Sigma Aldrich Chemie BV, The Netherlands) was selected for the enzymatic hydrolysis of cellulose and hemicellulose present in the fecal carbohydrate fraction. It is a widely available commercial multi-enzyme complex derived from *Aspergillus sp.*, which has a key activity as an endo-1,3(4)-β-glucanase, and side activities including arabinase, cellulase, hemicellulase and xylanase. According to the certificate of analysis, Viscozyme® L had an activity of 112 fungal beta-glucanase units/g, a protein content 70 g/kg and a density of 1.2 g/mL. The optimal conditions for hydrolysis as given by the supplier is a pH range of 3.3 -5.5 and a temperature range of 40-50 °C.

In a first set of experiments, the optimal incubation conditions for the enzyme treatment was investigated. The goal was to optimize enzyme loading, pH and temperature of the incubation (see Table 5.2 and 5.3). As a control for cellulase activity, an additional incubation was prepared with 1.53% sodium carboxymethylcellulose (CMC, 8% sodium, low viscosity, Sigma-Aldrich, Zwijndrecht, The Netherlands). Subsequently, the resulting optimal combination of incubation conditions was tested to have a benchmark yield for comparison with practical conditions in further experiments. Before each incubation, dried and ground fecal waste was suspended in demineralized water to create a standardized stock solution for the assays (6.1% w/V). Feces collected from the two different flow-through tanks were incubated separately, each incubation was done in triplicate, resulting in a total number of six replicates per incubation. Hydrolysis assays were carried out in 2 mL test vials, with a total incubation volume of 1.5 mL. To correct hydrolysis yields for the endogenous release of sugars from the fecal waste, negative controls without enzyme addition were incorporated for each incubation. Fecal dry matter content in the vials was 4.7% (w/V) throughout all incubations. Enzyme loading (EL) was always expressed as g protein per g fecal dry matter (% w/w). The pH in the vials was stabilized by using 200 mM acetate or phosphate buffer, depending on the required pH range (Table 5.3). NaN_3 (0.02% w/V) was used to prevent microbial degradation of the free sugars after hydrolysis (MacKenzie et al., 1985; Patterson and Watts, 2003b). All incubations were prepared on ice, and then incubated in a temperature controlled shaking water bath (200 rpm, Julabo SW22, Julabo USA Inc., Allentown, USA). To stop the enzymatic reaction after the incubation time, the vials were placed in a boiling water bath for 15 min. Then, the samples were centrifuged (10 min, 4500 rpm) and the supernatant was collected for further analysis. Saccharification of fibers was measured as total reducing sugars using p-hydroxybenzoic acid hydrazide (PAHBAH). Sugar liberation was determined as glucose equivalents, according to the standard curves obtained with glucose (Lever, 1972).

The benchmark incubation was performed without the addition of NaN_3. Furthermore, the hydrolysis products in supernatant of the benchmark incubation were analyzed by high-performance anion-exchange chromatography (HPAEC). HPAEC was used to detect

monomers of arabinose, rhamnose, galactose, glucose, xylose, mannose, fructose, fucose and galacturonic acid (MGalA) in the supernatant. The HPAEC analyses were performed at the department of Agrotechnology & Food Sciences (Wageningen University and Research Centre, Wageningen, The Netherlands). Post column addition was performed on an ICS-5000 unit (Dionex, Sunnyvale, CA, USA) using a CarboPac PA1 column (2 x 250 mm). Samples were eluted isocratically in 30 min with Millipore water, and the following elution profile was applied: 30.45 min, 0.1 M NaOH.0.4 M NaOAc in 0.1 M NaOH; 45.50 min, 1 M NaOAc in 0.1 M NaOH; 50.58 min, 0.1 M NaOH; 58.73 min, Millipore water (equilibration). A flow of 0.1 ml/ min 0.5 M NaOH was added postcolumn allowing pulsed amperiometric detection.

Table 5.2: Experimental design for different hydrolysis assays. Fecal waste from each flow-through tank was tested separately, and each incubation was tested in triplicate. The enzyme loading is expressed as % fecal dry matter (w/w).

	Temperature	**pH**	**Incubation time**	**Loading**
Time x loading	50 °C	5	0, 1, 3, 6, 24 h	0.5, 1.1, 2.1, 4.3%
pH range	50 °C	4.4, 5.1, 5.8, 6.7, 7.6	2 h	2.1%
Temperature	15, 25, 35 °C	5	2 h	2.1%
Benchmarking	50 °C	4.4	24 h	2.1%

Table 5.3: General recipe for incubations. For the negative controls, the volume of enzyme was substituted with demineralized water.

	Volume	**Concentration in vial**
Dried feces, suspended in demin. water	1150 µl	4.7% (w/V)
Viscozyme® L stock solution	50 µL	0.5 – 4.3% (w/w), depending on experiment
NaN_3	150 µL	0.2% (w/V)
Buffer	150 µL	200 mM (acetate buffer, phosphate buffer for pH 5.8 – 7.6)
Total Volume	1500 µL	

5.2.2 RAS experiment

The goal was to determine whether enzymatic pre-digestion of fecal waste can increase the degradability of fibers in a denitrification reactor in RAS, and thus increase the release of carbon from the fecal carbohydrate fraction. This experiment was part of a larger experiment, which was originally designed to solve two separate hypotheses, of which only one is presented here. The results concerning the COD and N balances and the degradation of fibers in a denitrification reactor using untreated fecal waste, are presented in Chapter 4 and are used here only as reference. The experiment was approved by the Ethics Commission for Animal Experiments of Wageningen University (Reference number 2013016.c).

RAS layout and experimental procedure

We used a total of six small-scale RAS, two served as control systems without denitrification, two systems used untreated fecal waste as carbon source for denitrification and two systems were supplied with enzymatically treated fecal waste as carbon source for denitrification.
The basic lay-out of all six RAS consisted of a circular fish tank (V=300 L), a settling unit for solids collection (V=75 L, HSL: 150 $m^3/m^2/d$) with a collection bottle connected to the bottom, a pump sump (V=75 L) and two trickling filters with cross-flow medium (V=0.059 m^3, A= 15.8 m^2 each). Two systems were kept in this configuration as controls, and four systems were operated with upflow-sludge blanket denitrification reactors (V= 10.5 L, upflow velocity 0.53 m/h). In RAS using untreated fecal waste for denitrification, a peristaltic pump continuously pumped settled solids from the feces collection bottle into the reactor at ~57 mL/min. A detailed description of the components and operating conditions of the control systems and systems using untreated fecal waste for denitrification can be found in Chapter 4. The recirculation systems, which were using enzyme treated fecal waste for denitrification, were additionally equipped with a mixing tank (V=30 L) before the denitrification reactor (Figure 5.1). Enzyme-treated feces were added into the mixing tank, and a peristaltic pump pumped the water/feces mixture constantly into the denitrification reactors (~55 mL/min). The mixing tank was airtight and stirred with a magnetic stirrer. A hose connected the mixing tank with the pump sump, so that the mixing tank was constantly refilled with system water from the pump sump. At the beginning of the experiment, each system was stocked with 25 rainbow trout of ~110 g. During the 42 days of experiment, the fish were fed at a restricted ration of 1.3% body weight per day.

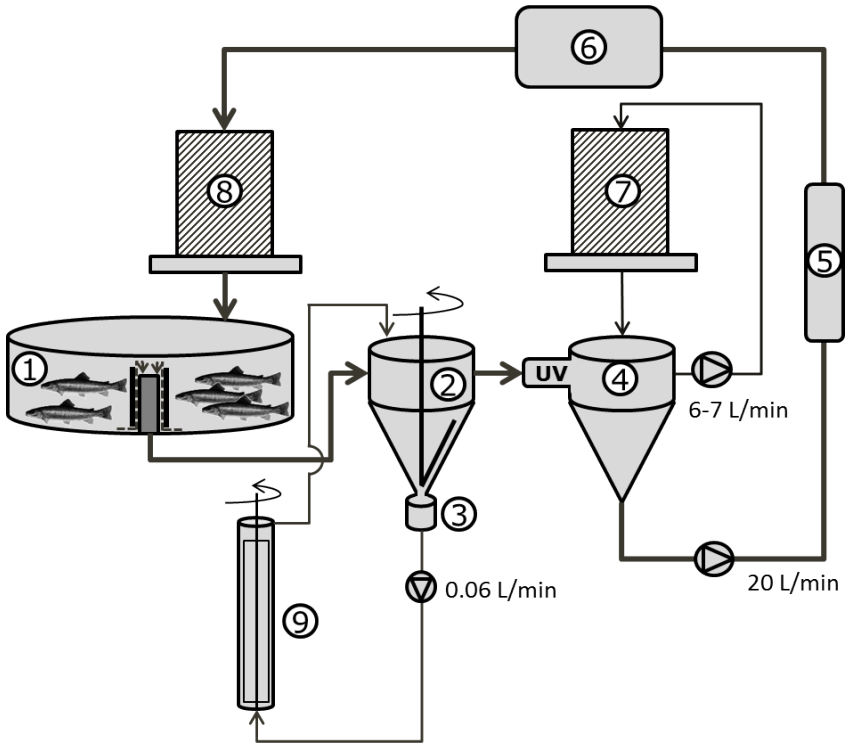

Figure 5.1: RAS layout for systems using enzyme-conditioned fecal waste, adapted from Meriac et al. (2014)(Chapter 2). [1] fish tank with double stand pipe (V= 300L, A= 0.72 m^2), [2] settling tank w/ stirrer to clean tank walls every 30 min (V=75L, HSL: 150 $m^3/m^2/d$); [3] Collection bottle connected to settling tank, cooled to 4 °C in control systems for feces collection (V= 250 ml); [4] Sump (V= 75L) and UV (UV-C 36 W, Phillips, Eindhoven, The Netherlands), water exchange and sampling point; [5] flow meter; [6] cooler-heater (TC20, Teco, Ravenna, Italy), [7] &[8] trickling filter w/ cross flow medium (V=0.059 m^3, A= 15.8 m^2 each), [9] upflow-sludge blanket denitrification reactor w/ stirrer (V_{active}= 10.5 L, HSL: 12.8 $m^3/m^2/d$), connected to the mixing tank (V=30 L).

Solids collection and enzymatic conditioning

Fecal waste was collected in the control systems twice a day before feeding, decanted, and stored at -20 °C until analysis. Fecal waste in the denitrification systems was directly pumped from the collection bottles at the bottom of the solids collectors into the denitrification reactors. Fecal waste for enzymatic conditioning consisted of a pooled 24 h sample of collected feces. The fecal waste collected in the afternoon during the previous day was stored on ice until the next morning, and mixed with the fecal waste collected in the morning. The composite sample of fresh fecal waste was directly incubated under the following conditions: V= 0.5 L, DM= 2.3%, pH= 4, T= 50 °C, EL=2.2 %, t= 24 h. The total volume of 0.5 L was adjusted by diluting feces with system water of their respective system. The pH was adjusted to 4 with HCl before enzyme addition. Residual oxygen was removed by gently sparging the incubation with N_2, then the bottles were closed air-tight and incubated for 24 h in a shaking water bath. The pH of the incubations was adjusted to ~7 with NaOH after the end of the incubation period. A 1.5 mL sample of the supernatant of the incubations was taken every 2-3 days to determine reducing sugars with a PAHBAH assays. The incubated feces were added once a day (11:00) to the mixing tank of the denitrification reactor. The chemical oxygen demand (COD) of the enzyme mixture was 457.3 g COD/L (measured according to APHA 5520 D, 1998). Based on one kg of feed, approximately 60 mL of enzyme were necessary for the treatment of the collected feces.

The total solids collected in denitrification systems were composed of excess sludge and residual solids. Excess sludge was collected when sludge bed height exceeded 1.4 m, and stored in aluminum trays at -20 °C as described in Chapter 4. The reactor residue was collected at the end of the experiment. All solids remaining in the reactors and/or mixing tanks were collected, and stored at -20°C for later analysis. After the experiment, all solids were pre-dried at 70 °C, ground and analyzed for dry matter, crude protein, crude fat, crude ash, acid-insoluble ash, energy, starch, neutral detergent fiber, acid detergent fiber, and acid detergent lignin, as described in Chapter 4. Furthermore, sugar monomers were measured before and after hydrolysis with HCl using HPAE-PAD (high performance anion exchange chromatography with pulsed amperometric detection) to determine hemicellulose content (xylose, mannose, arabinose, galactose, rhamnose and fucose; Saha, 2003).

5.2.3 Calculations and statistics

The main calculations are summarized in Table 5.4, further calculations on fish performance, apparent digestibility etc. can be found in Chapter 4. The treatments in the RAS experiment were compared using a t-test, assuming unequal variances (n=2). Statistical analyses were performed with SPSS (IBM SPSS statistics, version 20).

Table 5.4: Calculations. S: reducing sugars, DM: dry matter, ADC: apparent digestibility, determined in control systems, CP: crude protein, CF: crude fat, CA: crude ash, SWR: solid waste recovery, determined in control systems (Chapter 4); RS: residual solids, St: starch, C: cellulose, HC: hemicellulose, L: lignin. FG: feed given during experiment, X: solids constituents (fat, starch, cellulose, etc., in %)

Parameter	Unit	Formula
Reducing sugars due to hydrolysis (S_h)	[mg/mL]	$S_h = S_{treatment} - S_{neg.\ control} - S_{enzyme}$
Dry matter hydrolysis (H_{DM})[a]	[%]	$H_{DM} = (S_h * 0.9)/DM_{incubation} * 100\%$
Nitrogen-free extract in solids (NFE)	[g/kg DM]	NFE= DM- CP - CF - CA
Remainder in solids (R)	[g/kg DM]	R= NFE - St - C - HC - L
NFE hydrolysis (H_{NFE})	[%]	$H_{NFE} = (S_h * 0.9)/NFE_{incubation}$
Feces supplied to reactor (FS)	[g/kg feed DM]	$FS = FG * (100\text{-}ADC_{DM}) * SWR$
Degradability in reactor (D_R)	[%]	$D_R = (FS * X_{FS})/(RS * X_{RS}) * 100\%$

[a] according to Ghose (1987)

5.3 Results

5.3.1 In-vitro assays

Pre-experiments: The hydrolysis curves obtained with different enzyme loadings showed a high initial rate of product formation, which leveled off after ~3 h (Figure 5.2). Differences between treatments were clearly visible by eye in the incubations (Figure 5.2). After 24 h, the 0.5% enzyme loading yielded 53% of the maximal sugars observed in the 4.3% enzyme loading (Table 5.5). The data suggest that enzyme loading is more important than incubation time for maximal hydrolysis, since ~56% of the sugars were already released after 1 h of incubation (Figure 5.2). The control with CMC showed that only ~1.3% of the supplied CMC was converted to reducing sugars (Figure 5.2). As expected, hydrolysis efficiency was most sensitive for changes in pH. The hydrolysis yield at a pH 7.6 was only 4% of the maximum yield observed at a pH of 4.4 (Table 5.5). Using the yield at a temperature of 50 °C from the pH experiment (pH 5.1) as a reference, the yields in the incubations at 25°C and 15°C were 53% and 38% of the reference, respectively (Table 5.5).

Benchmark incubation: Although no sterilization was used, the final incubation yielded 82% of the yield observed under similar conditions in the "time x loading"- incubation (Table 5.5). This indicates that at least 22% of the NFE fraction was hydrolyzed. Analysis of the supernatant revealed that hemicellulose monomers and glucose made up 63% and 28% of the total monomers detected (Table 5.6). The total monomer content of 8.4 ± 0.47 mg/mL determined by HPAEC was higher than the total reducing sugar content of

7.0 ± 0.7 mg/mL determined by the PAHBAH assay. This can be explained by a lower response of the PAHBAH assay to some of the (hemicellulose) monomers (Lever, 1972).

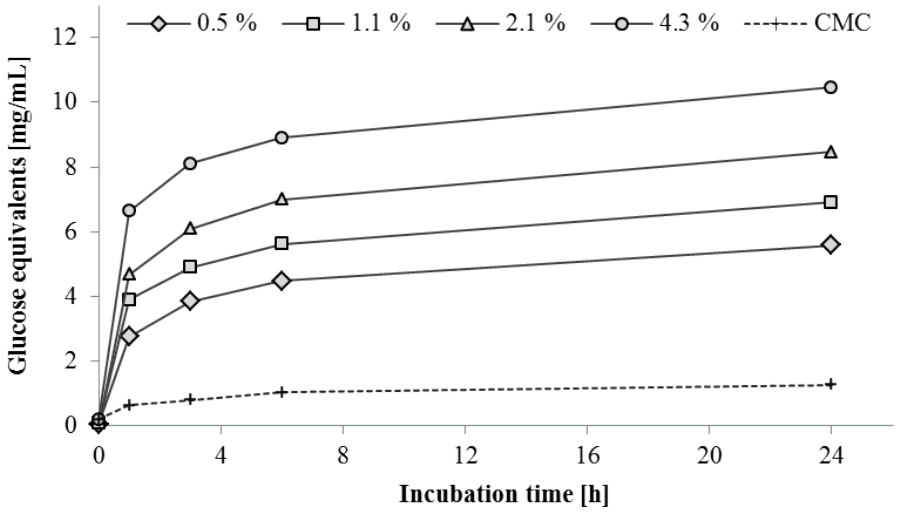

Figure 5.2: Reducing sugar generation in the time x loading incubations, including a CMC control (n=6). The NaCMC concentration in the incubation was 1.53%, using the same amount of enzyme as the 4.3% loading.

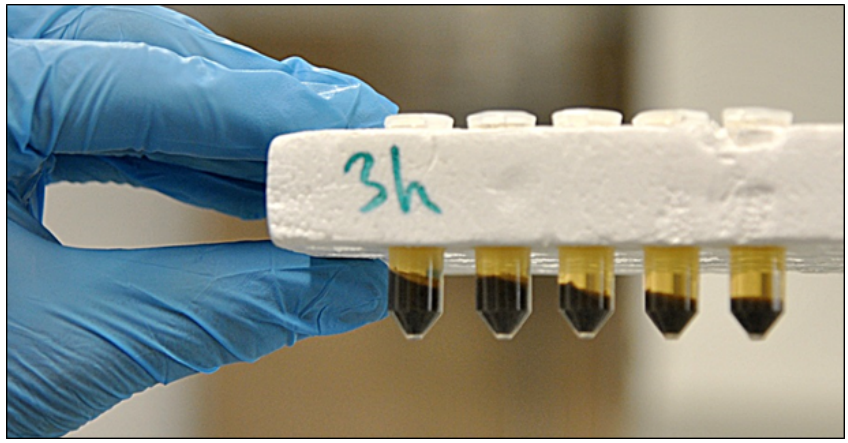

Figure 5.3: Time x loading incubations, samples after 3 h incubation time. The picture shows the samples after incubation and centrifugation. The corresponding loading of the vials left to right: 0%, 0.5%, 1.1%, 2.1% and 4.3%.

Table 5.5: Overview of incubations (mean ± standard deviation, n=6). For the time x loading experiment, only the results for t=3 and t=24 are shown. Hydrolysis is expressed as the release of glucose equivalents, and the corresponding hydrolysis of fecal dry matter (DM) and nitrogen-free extract (NFE). NFE is used to estimate the carbohydrate content in feces, and was calculated as NFE= DM-crude protein-crude fat-crude ash.

	Incubation conditions				**Hydrolysis**		
	Time [h]	**Loading** [% w/w]	**pH**	**T** [°C]	**Glucose equiv.** [mg/mL]	**DM** [%]	**NFE** [%]
Time x loading[a]	3	0.5	5	50	3.8 ± 0.5	7.4	12.3
	3	1.1	5	50	4.9 ± 0.7	9.4	15.7
	3	2.1	5	50	6.1 ± 0.8	11.8	19.5
	3	4.3	5	50	8.1 ± 1.2	15.6	26.0
	24	0.5	5	50	5.6 ± 0.5	10.8	17.9
	24	1.1	5	50	6.9 ± 0.8	13.3	22.2
	24	2.1	5	50	8.5 ± 1.1	16.3	27.1
	24	4.3	5	50	10.5 ± 1.8	20.2	33.6
pH[a]	2	2.1	4.4	50	5.0 ± 0.7	9.6	15.9
	2	2.1	5.1	50	4.5 ± 0.2	8.6	14.3
	2	2.1	5.8	50	3.2 ± 0.3	6.2	10.3
	2	2.1	6.7	50	0.8 ± 0.2	1.5	2.5
	2	2.1	7.6	50	0.2 ± 0.1	0.4	0.6
Temp.[a]	2	2.1	5	35	3.7 ± 0.3	7.2	12.0
	2	2.1	5	25	2.4 ± 0.1	4.7	7.8
	2	2.1	5	15	1.7 ± 0.3	3.3	5.5
Benchmark incubation[b]							
	24	2.1	4.4	50	7.0 ± 0.7	13.4	22.3
RAS incubation[c]							
	24	2.2	4	50	1.6 ± 0.8	6.1	10.1

[a] Dried feces collected in flow-through, 4.7% DM in incubation, 0.2% NaN_3, n= 6; [b] similar as in [a], except that no NaN_3 was used, n=4; [c] fresh feces collected in RAS, 2.2% DM in incubation, no NaN_3, n=40

Table 5.6: Monomer concentrations in benchmark incubation (mean ± standard deviation, n= 4).

Monomers		**[mg/mL]**
Hemicellulose	Arabinose	2.38 ± 0.06
	Fucose	0.12 ± 0.01
	Galactose	1.51 ± 0.07
	Mannose	0.38 ± 0.03
	Rhamnose	0.27 ± 0.01
	Xylose	0.63 ± 0.03
Others	Glucose	2.39 ± 0.11
	Fructose	0.29 ± 0.13
	M GalA	0.43 ± 0.44

5.3.2 RAS experiment

RAS using enzyme-treated feces showed a significantly lower nitrate concentration in the system water when compared with RAS using untreated feces (Table 5.7). Furthermore, no significant differences in fish performance were observed between treatments (Table 5.8). No significant differences were found between the proximate composition of the feces collected in flow-through and the feces collected in RAS (p> 0.05, Table 5.9).
The residual solids collected in the denitrification reactors showed no significant differences in fiber composition between reactors using treated or untreated fecal (Figure 5.4). The degradability of the fecal carbohydrate fraction (NFE) in the denitrification reactors was ~50%, showing also no significant differences between treatments (Table 5.10). Thus, enzyme treatment did not result in an additional liberation of carbon from the fecal fiber fraction. Analysis of the enzymatically treated fecal waste after 24 h of incubation shows, that at least ~10% of the NFE fraction was present as reducing sugars in the supernatant (Table 5.5).

Table 5.7: Average water quality in RAS (mean ± standard deviation, n=2). The results for the denitrification system using untreated fecal waste has been previously presented in Chapter 4.

		RAS with denitrification		
		Enzyme-treated feces	**Untreated feces**	***p***
T	[°C]	15.3 ± 0.24	15.6 ± 0.02	0.340
pH		7.3 ± 0.04	7.4 ± 0.03	0.156
O_2	[mg/L]	9.0 ± 0.03	8.9 ± 0.08	0.310
Conductivity	[mS/cm]	2.4 ± 0.04	2.4 ± 0.01	0.238
TAN	[mg/L]	0.1 ± 0.03	0.4 ± 0.13	0.220
NO_2-N	[mg/L]	0.2 ± 0.07	0.7 ± 0.21	0.184
NO_3-N	[mg/L]	108.9 ± 1.88	98.5 ± 0.96	0.041
tCOD	[mg/L]	26.4 ± 6.35	49.3 ± 1.13	0.114
Water exchange	[L/kg feed DM]	194.8 ± 1.48	188.2 ± 0.55	0.069

Table 5.8: Fish performance in RAS (mean ± standard deviation, n=2). FCR: feed conversion ratio, SGR: specific growth rate. The results for the denitrification system without enzymes has been previously presented in Chapter 4.

		RAS with denitrification		
		Enzyme-treated feces	**Untreated feces**	***p***
Initial body weight	[g]	112.3 ± 1.1	115.3 ± 2.8	0.353
Final body weight	[g]	182.0 ± 2.4	185.3 ± 2.7	0.327
Survival	[%]	100	100	n.a.
Feeding rate	[%/g/d]	1.28 ± 0.01	1.25 ± 0.02	0.325
FCR	[kg FW/kg DM]	1.10 ± 0.02	1.09 ± 0.00	0.773
SGR	[%/d]	1.15 ± 0.01	1.13 ± 0.02	0.429

Table 5.9: Feed and feces composition, as analyzed. Different batches of feed were used between the experiments. In vitro: feed and feces collected in flow-through tanks, RAS: feed and feces collected in RAS during degradation experiment (feces were collected in controls without denitrification), n=2, mean ± standard deviation, these results has already been presented in Chapter 4, NFE: nitrogen-free extract (calculated), NSP: non-starch polysaccharides (calculated), remainder: calculated as Remainder= NSP- Cellulose-Hemicellulose- Lignin, n.a.: not analyzed.

		Feed		**Feces**		
		In-vitro	**RAS**	**In-vitro**	**RAS**	***p***
Crude protein	[g/kg DM]	409.3	409.2	128.8 ± 2.9	127.5 ± 2.2	0.664
Crude fat	[g/kg DM]	132.4	129.3	22.0 ± 0.3	24.2 ± 1.3	0.253
Crude Ash	[g/kg DM]	95.3	95.9	247.2 ± 2.7	241.7 ± 0.9	0.184
NFE	[g/kg DM]	363.0	365.6	602.0 ± 5.9	606.6 ± 2.6	0.452
Starch	[g/kg DM]	n.a.	150.2	n.a.	23.6 ± 0.3	
NSP	[g/kg DM]	n.a.	215.4	n.a.	583.1 ± 2.2	
Cellulose	[g/kg DM]	n.a.	48.0	n.a.	205.5 ± 10.8	
Hemicellulose	[g/kg DM]	n.a.	57.5	n.a.	144.9 ± 7.4	
Lignin	[g/kg DM]	n.a.	24.0	n.a.	110.8 ± 5.2	
Remainder	[g/kg DM]	n.a.	86.0	n.a.	121.9 ± 0.5	

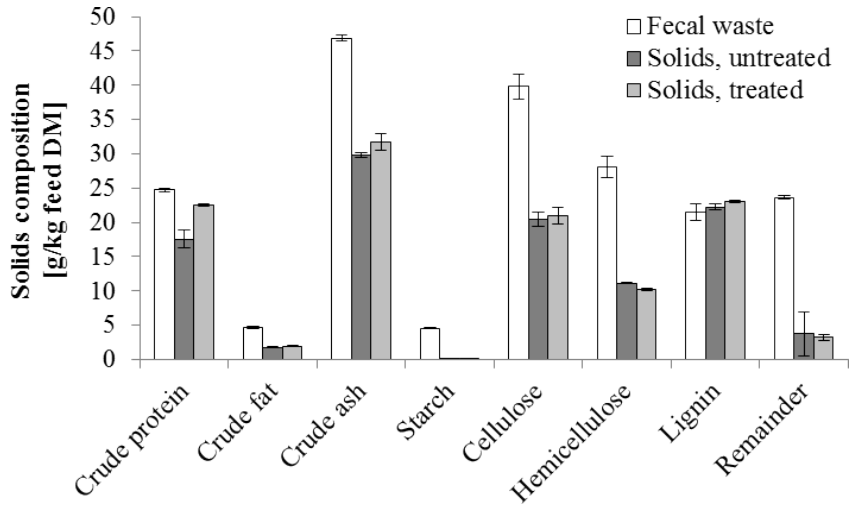

Figure 5.4: Dry matter composition of fecal waste and residual solids collected in denitrification systems. The composition of the solids collected in denitrification system without enzyme treatment has been partially presented in Chapter 4.

Table 5.10: Digestibility and degradability of fibers and hemicellulose monomers (mean ± standard deviation, n=2). The results showing the degradability of untreated feces in the denitrification systems has been partially presented in Chapter 4. NFE: nitrogen-free extract, DM: dry matter.

		Feces		
	Feed [%]	**Untreated [%]**	**Treated [%]**	***p***
Hemicellulose	51.1 ± 2.8	60.3 ± 0.1	63.6 ± 0.6	0.083
Arabinose	48.5 ± 3.1	76.3 ± 1.5	75.3 ± 0.2	0.500
Rhamnose	n.a. ± n.a.	52.4 ± 0.5	46.2 ± 1.9	0.122
Galactose	69.7 ± 2.0	78.7 ± 4.9	81.6 ± 0.1	0.552
Xylose/Mannose	37.2 ± 2.3	36.4 ± 1.2	43.1 ± 3.6	0.206
Fucose	59.0 ± 2.3	67.9 ± 9.4	74.3 ± 5.9	0.514
NFE	67.8 ± 0.3	50.9 ± 2.2	51.1 ± 0.4	0.930
Starch	97.0 ± 0.1	96.4 ± 1.3	96.5 ± 1.4	0.974
Cellulose	17.0 ± 3.9	48.7 ± 2.8	47.4 ± 3.2	0.719
DM	80.6 ± 0.1	44.8 ± 2.2	41.3 ± 0.9	0.232

5.4 Discussion

Based on the results of the CMC control, we conclude that Viscozyme® L has a low cellulolytic activity. This observation was confirmed by the results of the monomer analysis of the benchmark incubation, which suggests that most of the reducing sugars resulted from hemicellulose hydrolysis. Our findings suggest that a combination of Viscozyme® L and a cellulase is necessary to ensure the hydrolysis of lignocellulosic material in fecal waste, which is in line with results obtained in other studies (Foster et al., 2001; Hernández-Salas et al., 2009; Srichuwong et al., 2009; Tengerdy et al., 1991)
The maximal hydrolysis of the fecal waste in the pre-experiments was 20% DM, or 33% of the NFE fraction (EL: 4.3%, t= 24 h). Hydrolysis values in literature are 15% in poplar chips (Grous et al., 1986), 20.7% in pot-milled rice hulls (Mandels et al., 1974) and 52.2% in dried and pot milled rumen fibers (Mandels et al., 1974). However, a meaningful comparison of hydrolysis efficiencies across different studies is virtually impossible due to differences in enzyme and substrate properties for each study. Effective hydrolysis of lignocellulosic material (>90% saccharification) can usually only be achieved by pre-treatment of the lignocellulosic material (Alvira et al., 2010; Yang and Wyman, 2008).
We also observed that differences in enzyme loading had the most pronounced effect on the initial reaction rates, which is consistent with literature (Sattler et al., 1989; Wen et al., 2004). This in turn suggests, that longer incubation times can not necessarily

compensate for lower enzyme loadings. Sattler et al. (1989) were able to discriminate between fast and slowly hydrolysable cellulose fractions in their substrate, indicating that cellulase activity is decreased by differences in adsorption properties, pore size or accessibility. However, a decreasing enzymatic activity after the initial phase could also be explained by [i] end-product inhibition, [ii] precipitation or irreversible bonding of the enzymes to lignin or cellulose, or [iii] inactivation due to thermal effects (reviewed by Lynd et al., 2002). The benchmark incubation using an enzyme loading of 2.1% showed that approximately 22% of the NFE fraction was hydrolyzed within 24 h, which is in line with the results of the pre-experiments.

Since hydrolysis was severely hampered at a neutral pH, enzymatic conditioning of fecal waste in RAS would require a dedicated step including pH control for enzymatic digestion. However, the need for pH control seems to be detrimental to one of the major advantages of denitrification on internal carbon sources. Denitrification produces alkalinity, which can help to reduce the total alkalinity demand for nitrification in RAS biofilters (van Rijn et al., 2006). Although the initial degradation of solid waste and the production of volatile fatty acids (VFAs) could lower the pH (Suhr et al., 2013), it is not clear to which extend pH control can be minimized to still ensure good reactor and system performance. Therefore, the possibility to integrate enzymatic conditioning in existing designs using a pre-digestion step for the solids (as e.g. described by Suhr et al. 2013, Aboutboul et al. 1995 or Tal et al. 2009) remains yet to be explored.

The pre-digestion of fecal waste with enzymes did not have a significant effect on the degradation of fiber in the denitrification reactors. The lower nitrate concentrations in RAS using enzyme-treated feces for denitrification cannot be explained by a higher carbon liberation from fibers. This difference is probably an effect of [i] the enzyme as an additional carbon source for denitrification (27 g COD/kg feed DM), and [ii] a more efficient use of COD due to the batch-wise addition of solids (less oxidative COD losses). Approximately 50% of the carbohydrates (NFE) in fecal waste were degraded within the denitrification reactors, whereas we observed that only 22% of the fecal NFE was converted into reducing sugars in the benchmark incubation. Although the used enzyme did not show sufficient cellulolytic properties, we would still have expected to see an increased fiber degradability in the reactors. Grethlein (1985) stated, that the hydrolysis of hemicellulose can facilitate cellulose hydrolysis, by increasing pore size and the contact surface for cellulases. The hydrolysis of hemicellulose is often an important pre-treatment step to increase the degradability of lignocellulosic material (Chandra et al., 2007; Kumar and Wyman, 2009). Because no control incubation was performed with fresh feces using NaN_3 to suppress microbial activity, we can only assume that the hydrolysis efficiency with fresh feces was comparable to the results of the benchmark incubation. The lower reducing sugar content in the unsterilized sample can be explained by the conversion of free sugars into volatile fatty acids (VFAs), which can be readily used as a carbon source for denitrification (Aboutboul et al., 1995). This scenario seems likely, since [i] the fecal waste can provide a good inoculum for VFA generation (Leenhouwers et al., 2008), and

[ii] the incubation conditions actually resemble the conditions for thermophilic anaerobic digestion (50- 55 °C, Tchobanoglous et al., 2004). Suhr et al. (2013) observed that the generation of VFA from incubated sludge samples was the highest during the first 24 h of incubation at a temperature of 20 °C. Since no additive effect of the enzyme treatment on residual solids composition was observed, further degradation is probably hampered by structural and molecular properties of the remaining lignocellulosic material. This recalcitrant material would thus be an ideal starting point for the screening of enzymes, which can be effectively used to hydrolyze the problematic carbon fractions of especially low bioavailability for denitrification. However, if fecal waste is used as a substrate for screening enzymes, the enzymes should be able to hydrolyze more than 50% of the cellulose and hemicellulose present. Although we were not able to increase carbon bioavailability for denitrification, this study can provide a framework for using enzymes to improve the quality of fecal waste for denitrification on internal carbon sources.

5.5 Conclusions

Although that fecal waste was partially hydrolyzed, no additive effects on fiber degradation in the denitrification reactors were observed. The actual degradation of carbohydrates in the reactor exceeded the in-vitro results by a factor two. The fiber fraction remaining in the reactors after degradation seems to be especially resilient to microbial degradation, and would thus be an ideal candidate to identify enzymes which are suitable to increase carbon bioavailability for denitrification.

5.6 Acknowledgements

We would like to thank Dr. Henk A. Schols for his advice on the enzyme assays and Jelle Busscher for his help with the denitrification experiment. Furthermore, we would like to thank Ronald Booms for this help during the methodology development. We would like to acknowledge Interreg IV A and Agentschap NL for funding and all contributors of the EM-MARES and AquaVlan project consortia.

6 General discussion

6.1 Main findings of this thesis

The results of this thesis show how the substitution of fish meal with unpurified plant ingredients can affect waste dynamics in RAS. The main findings can be organized within a concise conceptual framework, and are illustrated in Figure 6.1. Feed digestibility, feces recovery and feces degradability are the principal factors determining solid waste production, system performance and treatment potential with denitrification.

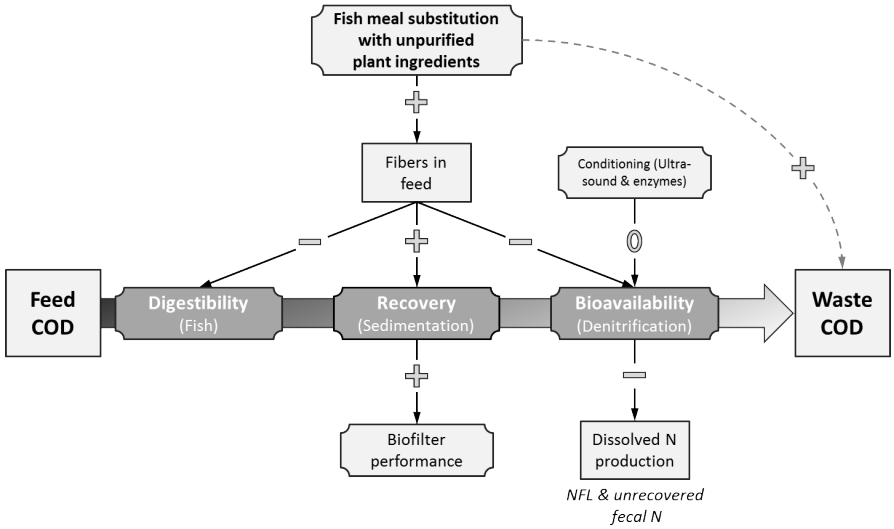

Figure 6.1: Main findings. The conceptual framework shows the effect of fibers on fecal COD production, recovery and degradability. Fibers originating from the feed decreased feed digestibility, increased solid waste recovery and decreased COD degradability. This decreased the degradable COD load on the biofilters, which is beneficial for the nitrification performance. Total COD waste production was higher for the high fiber diet than in the low fiber diet, even after applying denitrification.

Fibers lowered the dry matter digestibility of feed, and subsequently increased the production of fecal waste (Chapter 2). Based on digestibility data, the HNSP diet produced 50% more solid COD than the LNSP diet. The recovery of HNSP feces in

sedimentation was 40% higher than the recovery of LNSP feces. Despite an increased solid waste production, the combined effect of a higher recovery and lower degradability of HNSP feces resulted in a 55% lower BOD_5/N ratio in the biofilter load. Furthermore, fibers also increased the median particle size in fecal waste from 24 µm in LNSP feces to 101 µm in HNSP feces (Chapter 3). The increased production and recovery of HNSP feces also increased COD/N ratios in the waste stream from 3.4 for LNSP to 7.2 for HNSP (Chapter 2). Although a COD/N ratio of 7.2 would be theoretically sufficient for full nitrate removal in denitrification (Chapter 2), the bioavailability of fecal carbon was limited due to a high fiber content (Chapter 2, 3, 4). Approximately 40% of the hemicellulose and 50% of the cellulose from HNSP feces remained undegraded in the reactor (Chapter 4). The treatment of fecal waste with high intensity, low-frequency ultrasound or enzymes did not sufficiently increase the bioavailability of carbon (Chapter 3, 5). However, by using fecal waste as a carbon source for denitrification, we were able to reduce COD and N waste emissions from RAS by 50% for the HNSP diet (Chapter 4).

6.2 Methodological approach

The strength of this study lies in the combination of a bio-energetic nutrient mass balance on fish level with a nutrient mass balance measured on system level. The combination of both methods offers the advantage to determine system-inherent nutrient losses, which are relevant to estimate solid waste recovery, passive denitrification and organic loading on the biofilters. To our best knowledge, no other studies were present in literature, which applied both methods in one experiment.

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) was chosen as the model species, since RAS are often used for the production of carnivorous fish of high value (Dalsgaard et al., 2013; Martins et al., 2010). The current prospect is that RAS will be increasingly applied in the production of salmonids species as rainbow trout and Atlantic salmon (Dalsgaard et al., 2013). However, the obtained results cannot directly be extrapolated to other species, such as carp or tilapia. In these species, conditions can be very different due to general differences in feed formulation and possible hind-gut fermentation of carbohydrates (Amirkolaie et al., 2006).

The starting point for formulating the two experimental diets was the assumption, that fish meal substitution with plant-ingredients will not affect fish performance while introducing non-starch polysaccharides (NSPs) into the feed. Therefore, the two diets needed to be formulated iso-energetically and iso-nitrogenously (equal DP/DE ratios) to ensure similar fish performance and non-fecal loss (NFL) of nitrogen (N). The total amounts of digestible protein and energy in the experimental feeds were comparable with those in a commercial fish feed as described by Bureau and Hua (2010). The DP/DE ratios were chosen within the recommended range for rainbow trout of 18-24 kJ/g (Azevedo et al., 2004). In the LNSP diet, starch was the main source of digestible energy, while

having a low content of NSPs (~2% DM). In the HNSP diet, starch was substituted with NSPs (~19% DM), and the amount digestible energy was kept constant by increasing the fat content. Although the diets were not directly related to practical diets[1], the contrast in NSPs served the purpose to study the effect of changes in the dietary carbohydrate composition on the feed-fish-waste axis. Differences in waste production and COD/N ratios observed in the experiments should be thus only a reflection of differences in dietary carbohydrate composition.

In this study, the correct determination of the bio-energetic nutrient balance and metabolic waste production at the level of the organism (fish) is crucial. This requires accurate data on digestibility, which in turn hinges on a reliable method for feces collection. Since fecal waste was collected in RAS, biomass originating from the system (e.g. sheared biofilm) could have altered feces composition, thus compromising the correct determination of feed digestibility. However, no significant differences were found in ADC of dry matter, crude protein, crude fat, crude ash and NFE when comparing ADCs determined in RAS (Chapter 2) and flow-through (Chapter 3)($p > 0.05$, t-test, data not shown).

Since a constant water exchange was essential for our experiments, the use of drum filters could have caused problems due to varying backwash volumes (Dolan et al., 2013). Therefore, fecal waste was collected via sedimentation with overflow rates of 0.17 cm/s (RAS, Chapter 2,4,5) and 0.23 cm/s (flow-through, Chapter 3,5). According to Wong and Piedrahita (2000), this should have been sufficient to collect at least 87% of the fecal waste. The sedimentation properties of fecal waste are also highly relevant for dual drain systems, in which the solids are directly separated from the system flow by sedimentation at the bottom of the fish tanks[2].

In summary, we can assume that the choice of fish species, diet, model system and the applied methodology is suitable to investigate how an increased dietary fiber content can affect system performance and waste production in RAS.

6.3 The effect of fibers on the feed-fish-waste axis

Digestibility, recovery and degradability are the main factors affecting solid waste production and the treatment potential of denitrification on internal carbon sources (Figure 6.1). These three factors will be discussed to understand how the substitution of fish meal with unpurified plant ingredients will affect waste dynamics in RAS.

[1] However, the ingredients used for the HNSP diet are commonly used in commercial fish feeds, and crude fiber contents of 5% and above were also observed in commercial diets. Overall, the HNSP diet is probably closer to an average commercial feed formulation than the LNSP diet.

[2] Soilds separators in dual drain systems are often operated at comparable or even higher overflow rates (e.g. 0.31 cm/s, Davidson and Summerfelt, 2005; 1.16 cm/s, Summerfelt and Penne, 2005)

6.3.1 The effect of fiber on feed digestibility and waste production in fish

The results obtained in Chapter 2 and 3 show how unpurified plant ingredients can reduce feed digestibility, and thus increase the production of fecal waste in fish. These results are in line with the findings of other authors, who reported similar effects of NSPs in fish feeds (Farhangi and Carter, 2007; Glencross, 2009; Glencross et al., 2012). We assume that this trend should be consistent for all fish species, since even herbivorous fish show little capacity to digest fibers (Halver and Hardy, 2002). Bureau and Hua (2010) estimated, that the production of feeds with a dry matter digestibility >90% is economically not feasible. Therefore, the "best case"- scenario for solid waste production in aquaculture would be 100 g DM/kg feed DM. However, in our study the LNSP and HNSP diet produced 160 and 230 g DM/kg feed DM. The HNSP diet produced 44% more solid waste than the LNSP diet, and more than double the amount than the "best case"-scenario of Bureau and Hua (2010). This result confirms that the substitution of fish meal with unpurified plant ingredients will increase waste production in aquaculture. Solid waste management will become a more important issue for RAS in the future. For open systems, such as cages and raceways, this means that more solid waste will be discharged into the environment.

6.3.2 The effect of fibers on feces recovery and system performance

Fecal recovery

The HNSP diet resulted in an increased recovery of fecal waste when compared to the LNSP diet (79% for HNSP vs. 56% for LNS, Chapter 2). The fecal waste recovery of HNSP feces was exceptionally good in comparison to removal efficiencies of 50-60% found in literature (Couturier et al., 2009; Roque d'Orbcastel et al., 2008; Summerfelt and Penne, 2005). However, the removal efficiency of a solids separator is usually determined by comparing the concentration of total suspended solids (TSS) entering and leaving the unit (Dolan et al., 2013; Summerfelt and Penne, 2005; Timmons and Ebeling, 2007). Dalsgaard and Pedersen (2011) showed that 21% of the COD egested by the fish was directly converted to suspended/dissolved COD, and might therefore not be necessarily detected in TSS measurements. Therefore, our approach of digestibility-based determination of fecal recovery most likely underestimates the removal efficiency of the sedimentation units based on TSS-in/out.

Although some studies have shown that plant ingredients can decrease fecal waste recovery, this was usually related to purified plant ingredients (e.g. soy protein concentrate) (Brinker and Friedrich, 2012; Davidson et al., 2013). The good recovery of HNSP feces could be explained by the presence of non-starch polysaccharides originating from the unpurified plant ingredients, which act as binders and stabilize the fecal pellets (Amirkolaie

et al., 2005). In a Master thesis carried out within the framework of this PhD research, Stringer (2012) performed stability and sedimentation trials with HNSP and LNSP feces[3]. His results also suggest that the improved stability of HNSP feces is the main reason for an increased recovery rate.
Brinker et al. (2005b) have successfully demonstrated how fecal stability and recovery can be improved by the addition of NSPs (guar gum) to fish feeds. However, the role of ingredient-inherent NSPs as binders is not well described in literature (Amirkolaie et al., 2005). Gröner and Pfeffer (1997) have shown how ingredient selection can be used to improve feces stability in dogs, and that the fiber content alone is not a suitable indicator to predict feces stability. Ingredients rich in e.g. arabinoxylans could increase feces viscosity (Choct, 1997), which can improve feces stability and recovery (Amirkolaie et al., 2006; Brinker, 2007). Fish farmers and feed manufacturers alike could benefit from a more systematic research on the functional properties of fibers in fish feeds.
Furthermore, feces produced with the HNSP diet contained larger particles than feces produced with the LNSP diet (Chapter 3). HNSP feces contained a distinct fraction of particles between 200 and 500 µm, which were also very resilient to mechanical stress. Subsequently, the recovery of disintegrated fecal waste on a microscreen of 36 µm increased from 10% for the LNSP diet to 50% for the HNSP diet.
The increased stability of the fecal pellet combined with an increased particle size facilitates the recovery of HNPS feces in RAS by either sedimentation or microscreen filtration. However, an improved settleability of feces could exacerbate existing problems in salmon aquaculture by increasing the organic load on the sediments below sea cages (Reid et al., 2009).

Effect on system performance and biofilter load

As water exchange rates are being reduced in RAS, the accumulation of microparticles in the water is becoming a more important issue to ensure fish welfare (Chapman et al., 1987; Davidson et al., 2009; Fernandes et al., 2014; Patterson and Watts, 2003b). Particle size analysis of HNSP and LNSP feces revealed that both experimental diets produce the same amount of microparticles (<36 µm) per kg of feed (Chapter 3). These particles could pass a drum filter screen of 36 µm and accumulate in the system water, which in turn can impair fish welfare. However, due to a lower degradability of HNSP feces (Chapter 2), the microparticles produced with the HNSP diet should have a higher accumulation potential than microparticles from the LNSP diet. Patterson and Watts (2003a) also suggested that fibers originating from plant ingredients could substantially contribute to the microparticle load in RAS. Therefore, the effect of plant-based diets on microparticle generation and fish welfare in RAS should be addressed in future research.
Although the HNSP diet produced 50% more fecal COD than than the LNSP diet, the

[3]Fecal waste was produced and collected in flow-through tanks under similar conditions as described in Chapter 3.

total load of COD on the biofilters was ~30% lower due to an improved recovery of HNSP feces (Chapter 2). As a result of the low degradability of HNSP feces, the BOD_5/N ratio was almost half of the BOD_5/N load determined with the LNSP diet (Chapter 2). The effective organic load on the biofilters per kg of feed seemed to be determined only by the recovery efficiency of fecal waste in the solids removal unit, irrespective of diet composition (Figure 6.2). As the fiber fraction in feeds increases, the degradability of the fecal waste should decrease as the amount of fecal waste increases (Figure 6.3). This finding could suggest, that a BOD_5/N-ratio of 0.5 can be realized in RAS biofilters with a generic feed[4], if ~80% of the fecal waste can be removed. However, since a failure of the biofilter can result in a total loss of the fish stock, an ample safety margin in biofilter design is always advisable. The relation of fecal recovery and nitrification rate shows how fecal properties as degradability and recoverability are connected, and suggest that the recovery of fecal waste is the principal factor in determining biofilter performance.

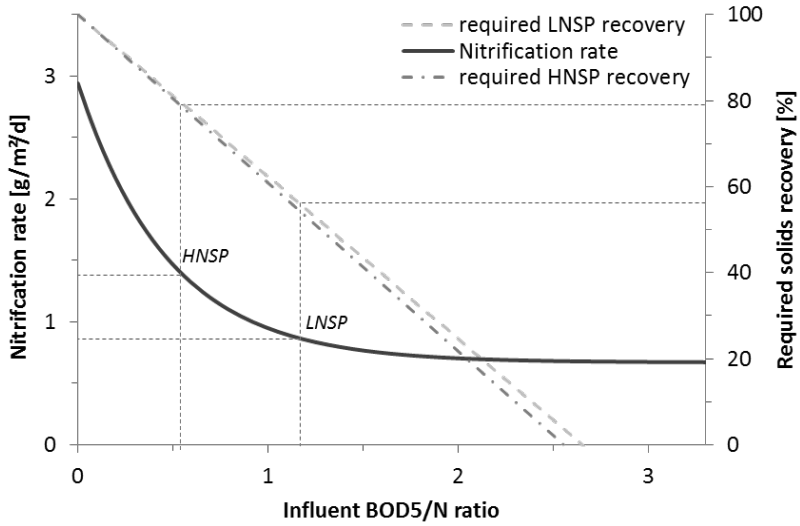

Figure 6.2: Relationship between fecal recovery and theoretical nitrification rate, assuming an average TAN production of 31 g N/ kg feed DM for both diets. The nitrification rate is based on Chen et al. (2006), correcting for the BOD_5/COD ratio of the substrate used in the original experiment of Ling and Chen (2005). The straight lines indicate the required recovery of a diet to achieve a certain BOD_5/N ratio and the corresponding nitrification rate. Changes in N production in fish would affect the slope of the lines for required recovery.

[4] Assuming a TAN production rate of ~30 g/kg of feed, and a similar DP/DE-ratio and ADC for crude protein, fat and starch.

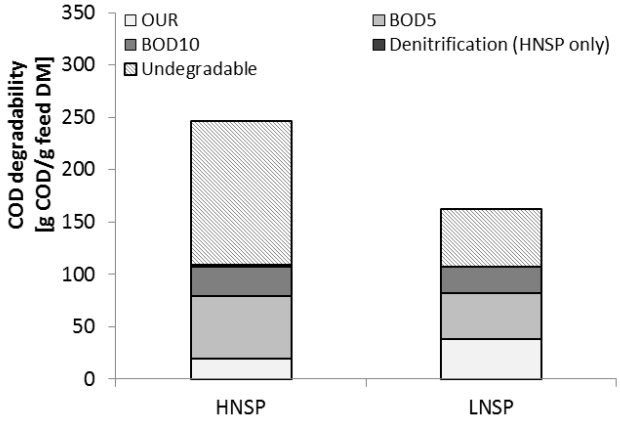

Figure 6.3: Total COD production and degradability. The degradable COD fraction shows how the degradability increases with incubation time/method, as determined in OUR tests (Chapter 3), BOD_5, BOD_{10} (Chapter 2) and in the denitrification experiment (only for HNSP diet, Chapter 4).

6.3.3 The effect of fibers on feces degradability and treatment potential of fecal waste

Degradability of fibers in denitrification

As expected, the inclusion of unpurified plant-ingredients had a negative impact on carbon bioavailability in feces throughout all experiments. Despite that the HNSP diet produced more COD than the LNSP diet, both diets produced similar amounts of degradable carbon (BOD_5, BOD_{10}) per kg of feed (Figure 6.3). The results of the OUR tests (Chapter 3) indicate that 35% of the total degradable COD in LNSP feces was utilized within 1-2 hours (Figure 6.3). In contrast, only 18% of the degradable COD was readily available in HNSP feces, representing just 8% of the total fecal COD. The high degradability of LNSP feces can be explained by a high starch content, which should also be a good substrate for denitrification. This was confirmed in Chapter 4, the denitrification reactors were able to degrade 96% of the starch present in the fecal waste. However, a high, sudden load of easily degradable feces into a denitrification reactor could also cause problems, such as: [i] an accelerated formation of N_2, causing the expansion of the sludge bed and a possible wash-out of biomass from the reactor (Lettinga et al., 1980), and/or [ii] a rapid depletion of nitrate, thus increasing the risk of producing unwanted by-products such as nitrite or H_2S in the reactor (Balderston and Sieburth, 1976). Thus, caution is advised if new feeds are introduced into a fish farm, being that a denitrification reactor can be very sensitive towards changes in dietary carbohydrate composition.

The utilization of cellulose as a substrate for denitrification is not a novelty by itself (Skinner, 1972), as cellulose or lignocellulosic material was often used as a carbon source for denitrification in e.g. drinking water (Lowengart et al., 1993; Rocca et al., 2007; Sobti and Sharma, 2011; Volokita et al., 1996). The denitrification reactors were able to degrade 44% of the total COD of HNSP feces, and 50% of the cellulose and 60% of the hemicellulose in HNSP feces. This seems to be in the range observed in literature for the anaerobic degradation of fibers (Table 6.1). The determined degradability of HNSP feces was almost identical between the BOD_{10}-tests (Chapter 2) and the denitrification experiment (Chapter 4)(Figure 6.3). This suggests that BOD_{10} can be used as a proxy to predict fecal COD degradability in denitrification. Based on the degradability coefficients as determined for HNSP feces (Chapter 4), one would predict a COD degradability of 62% for LNSP feces. This fits remarkably well with the results of the BOD_{10}-tests, which showed a degradability of 66 ± 2% in LNSP feces (Chapter 2).

Table 6.1: Overview of fiber degradability in literature. HC: hemicellulose, C: cellulose. L: lignin

Substrate	Conditions	Time	Temp.	Degradation	Reference
HNSP feces	Denitrification in RAS	42 d	15 °C	60% HC, 49% C, 0% L (51% total carbohydrates, 41% dry matter)	Chapter 4, this thesis
Wood chips	Denitrification reactor in RAS (+ external carbon source)	140 d	n.a.	16% mass loss	Saliling et al., 2007
Wheat straw				38% mass loss	
Wheat straw	Denitrification	40 d	25 °C	42% mass loss	Soares and Abeliovich, 1998
Municipal refuse	Anaerobic digestion	219 d	40 °C	46% HC, 42% C	Eleazer et al., 1997
Primary sludge from wastewater treatment plant	Anaerobic digestion	30 d	35 °C	72% carbohydrates	Mahmoud et al., 2004
Straw/manure 1:2	Composting, passive aeration	150 d	55-70 °C	100% HC, 64/70% C, 0% L	Eiland et al., 2001
20% cotton gin waste, 80% olive mill wastewater	Composting, on-demand ventilation	182 d	55 °C	74% HC, 75% C, 54% L	Paredes et al., 2002
11% maize straw, 89% olive mill wastewater		92 d		77% HC, 70% C, 20% L	

As cellulose and hemicellulose can be at least partially utilized in a denitrification reactor, this also means that the hydrolysis rate of fibers will limit the denitrification rate (Noike et al., 1985). To ensure sufficient nitrate removal in a denitrifcation reactor, longer sludge retention times might be required to compensate for a lower hydrolysis rate (Mahmoud et al., 2004).
Although we determined a sludge retention time of 9 days in the reactors, the actual incubation time of fibers in the reactor might have been even up to 42 days. The hemicellulose, cellulose and lignin remaining in the reactor after the experiment accounted for 93.5% of the collected total (data not shown). This shows that the removal of fibers from the reactor with the excess sludge was negligible. The question remains whether an extended sludge retention/incubation time could actually increase the total degradability of cellulose and hemicellulose (Table 6.1)(Eleazer et al., 1997). The degradability of lignocellulosic material is not only determined by the physical accessibility of the cellulose (particle size, porosity, hemicellulose/lignin content), but also by the ratio of crystalline to amorphous cellulose (Lynd et al., 2002; Zhu et al., 2008). Substrates with a high lignin content and high cellulose crystallinity will offer less contact points for cellulase adsorption and hydrolysis (Komilis and Ham, 2003; Teeri, 1997). Since lignin is virtually undegradable under anaerobic conditions (Eleazer et al., 1997; Komilis and Ham, 2003), a significant amount of carbon will remain as a recalcitrant fraction in a denitrification reactor (Shao et al., 2009). Thus, it is questionable that a prolonged incubation of fibers in the denitrification reactor could substantially increase the amount of carbon, which can be utilized in denitrification.
Due to the low degradability of fibers, also other "traditional" treatment methods (e.g. sludge digestion, composting) will most likely not be able to resolve the solid waste problem arising from an increasing dietary fiber content. Furthermore, the value of solid waste from aquaculture as fertilizer might decrease, as the relative content of nitrogen and phosphorus decreases proportionally to the amount of refractory organic carbon.

Increasing carbon bioavailability for denitrification

Fibers present in the fecal waste limit the bioavailability of carbon for denitrification. Thus, we face essentially the same challenge as other biotechnological processes such as biogas or biofuel production (Sun and Cheng, 2002; Taherzadeh and Karimi, 2008). In these processes, the application of pre-treatment methods is often necessary to ensure an efficient conversion of biomass into bioavailable carbon sources (Alvira et al., 2010). Correspondingly, we also tested whether some of the pre-treatment methods can resolve a limited carbon bioavailability in fecal waste.
The reasoning behind using ultrasound treatment was that a reduction in particle size could increase carbon bioavailability in fecal waste. Yet, ultrasound treatment did not sufficiently reduce particle size or increase carbon bioavailability in fecal waste of both

diets. Since most of the particles in the fecal waste were already smaller than 500 µm, it is questionable whether a reduction in particle size could actually improve the long-term degradability of fecal waste (Ambus and Jensen, 1997; Wen et al., 2004). Therefore, there seems to be no added value of using ultrasound to increase the long-term degradability of fecal waste in aquaculture.

Initially, we intended to use ultrasound for treating the excess sludge leaving the denitrification reactors, since it should contain significant amounts of microbial biomass. In denitrification, a part of the bioavailable carbon is converted into microbial biomass and will not be immediately used for nitrate removal[5](Tchobanoglous et al., 2004). To maintain sludge bed height, the excess growth needs to be removed from the reactor, thus removing a valuable carbon source for denitrification (Lettinga et al., 1980). Using ultrasound, the bacterial cells can be disintegrated and the liberated organic carbon re-used as a readily available carbon source for the reactor (Vaxelaire et al., 2008; Mao et al., 2004). The potential of using ultrasound to recycle the microbial yield, and thus to reduce the COD/N ratio necessary for denitrification, remains yet to be explored.

Almost 25% of the fecal carbohydrate fraction was successfully hydrolyzed after enzymatic digestion, despite an apparent lack of cellulolytic activity of the enzyme mixture (Chapter 5). The majority of the liberated sugar monomers resulted from the hydrolysis of hemicellulose, which can indirectly improve the degradability of cellulose (Chandra et al., 2007). However, enzymatic treatment of fecal waste did not increase the degradability of fibers in the reactors (Chapter 5). This indicates that the hydrolysis of fibers is not a linear process, and that factors as cellulose crystallinity and lignification can limit the final degradability of the substrate (Fan et al., 1980; Komilis and Ham, 2003; Laureano-Perez et al., 2005). These structural properties will hamper the hydrolysis efficiency of endogenous and exogenous enzymes alike (Rodriguez et al., 2005). Even so, the residue remaining in the reactors after digestion can still be used to screen for enzymes, which might be capable of attacking the recalcitrant fraction of cellulose and hemicellulose. Enzymatic pre-digestion of fecal waste could still be of interest if long sludge retention times are not feasible and/or if the reactor relies primarily on readily available carbon sources.

The applied conditioning methods did not resolve the limited carbon bioavailability in feces produced with the high fiber diet (Chapter 3, 5). The choice of alternative pre-treatment methods is rather limited for RAS, since toxic effects on fish/bacteria or a too low dry matter content can hamper feasibility (Alvira et al., 2010). However, the bioavailability of fibers for denitrification could also be increased by a smart selection of feed ingredients. To improve fiber degradability, feed ingredients with a low lignin content and low cellulose crystallinity should be used. The degradability of fibers in feed ingredients

[5]The effective microbial yield is also determined by the endogenous decay coefficient, which is determined by sludge retention time. The true demand of bioavailable carbon (bCOD) in the reactor can thus be described with the following formula: $bCOD/NO_3\text{-}N = 2.86/(1\text{-}1.42 \times yield)$(Tchobanoglous et al., 2004). However, a conversion of around 35% of the bioavailable carbon into bacterial growth is expected in a comparable set-up (SustainAqua, 2009).

could potentially even increase during extrusion (Karunanithy and Muthukumarappan, 2010; Kleinebudde et al., 2000).

6.3.4 The effect of fibers on denitrification and total waste production in RAS

The efficiency of a denitrification reactor in RAS is determined by the cumulative effects of diet composition on digestibility, recovery and degradability. Denitrification on internal carbon sources successfully controlled the accumulation of nitrate in the system, reduced COD and N emissions, and decreased alkalinity demand in RAS (Chapter 4)(Table 6.2). Since only the high fiber diet was tested in denitrification, the degradability of LNSP in denitrification needs to be estimated for comparison using BOD_{10} (p. 96). If denitrification was applied under the same conditions as described in Chapter 2, more nitrate would have been removed with the HNSP diet than with the LNSP diet. However, this is mainly the consequence of an increased fecal recovery, since the production of bioavailable carbon (BOD_{10}) per kg of feed was similar for HNSP and LNSP (Figure 6.3). Although more COD was produced with the HNSP diet, the quality of fecal waste as a carbon source diminishes. Thus, fibers do not have an added value as an additional carbon source for denitrification under conditions as described in this thesis. Nevertheless, denitrification on internal carbon sources is an effective tool to reduce solid/dissolved waste production and water demand in RAS. As nitrate levels can be controlled by denitrification, the accumulation microparticles or heavy metals can become the new bottleneck for the reduction of water exchange rates in RAS (Davidson et al., 2009).

Table 6.2: Calculated COD/N balances for HNSP and LNSP diet, with or without denitrification. Solid and dissolved waste production is based on the balance presented in Chapter 2. The bioavailability of carbon for denitrification was calculated for HNSP using the degradability determined in Chapter 4, whereas it was estimated for LNSP using BOD_{10} (Chapter 2). Nitrate removal was calculated using the biodegradable COD/N ratio for denitrification of 4.41 as determined in Chapter 4.

		RAS				Reduction	
		Conventional		Denitrification		by denit.	
Parameter	**Unit**	**HNSP**	**LNSP**	**HNSP**	**LNSP**	**HNSP**	**LNSP**
COD production[a]	[g/kg]	207	104	121	44	42%	58%
N production[a]	[g/kg]	31	30	11	18	63%	41%
Net $NaHCO_3$ demand[b]	[g/kg]	178	189	70	114	60%	40%
Min. water exchange[c]	[L/kg]	270	269	86	155	68%	42%

[a] including solid and dissolved waste, [b] assuming that water refreshment does not contribute to alkalinity. Alkalinity consumption and generation was calculated according to the SustainAqua Handbook (2009), and was based on the total nitrogen load on the system, taking the contribution of unrecovered and degraded feces into account; [c] water exchange necessary to maintain an NO_3-N concentration of 100 mg/L in the system.

Nonetheless, fibers could have other beneficial effects for the operation of denitrification reactors. The fibrous particles could act as nuclei for the formation of sludge granules, while providing a carbon source for the bacteria (Boley et al., 2000; Shao et al., 2009; Yoda et al., 1989). This could improve the settleability of the sludge bed, and reduce the wash-out of biomass from the reactor. Furthermore, more of the bioavailable COD could be used in denitrification since the granules will act as a diffusion barrier for oxygen (Chiu et al., 2007).
In Chapter 4, it was also observed that 54% of the degraded COD was actually not used for denitrification, but probably respired with the dissolved oxygen entering the reactor with the system water. Oxidative losses are a common problem for denitrification, which can be reduced by improving reactor design (Henze et al., 1997; Klas et al., 2006b; Müller-Belecke et al., 2013). Increasing the hydraulic retention time by decreasing the flow would decrease the amount of oxygen entering the reactor, resulting in an increased utilization of bioavailable carbon in denitrification. Thus, it is necessary to optimize the reactors for an efficient use the bioavailable carbon, instead of focusing only on increasing carbon bioavailability in fecal waste.

6.4 General recommendations

This study demonstrated the effect of fibers originating from unpurified plant ingredients on system performance and waste production in RAS. It also shows how diet formulation can be used to change waste dynamics along the feed-fish-waste axis. A selective choice of ingredients could be used to improve fecal waste recovery without the addition of external binders. This in turn can improve biofilter performance in RAS, or could even allow for using smaller solids separation units in the culture systems. The degradability of fecal waste can also be improved by choosing feed ingredients with a low lignin content and low cellulose crystallinity.
Although the topic of phosphorous was not addressed in this thesis, further research in this direction is strongly recommended. Phosphorus is a valuable resource and has a high eutrophication potential when discharged into the environment (Verdegem, 2013). The degradation of fecal waste will release phosphorus, which can increase the eutrophication potential of the dissolved waste stream (Conroy and Couturier, 2010). Therefore, RAS could greatly benefit from innovative solutions to recuperate phosphorus as products of value, such as struvite (Xavier et al., 2014).

6.5 Main Conclusions

The substitution of fish meal with unpurified plant ingredients imposes a new challenge on traditional waste management concepts in aquaculture. Fibers originating from unpurified plant ingredients reduce the dry matter digestibility of fish feeds, and thus increase solid waste production in aquaculture systems. However, fibers in the fecal waste can also improve solid waste removal, which is beneficial for the performance of nitrifying biofilters in RAS. The increased production of solid waste can be counteracted by using fecal waste as a carbon source for denitrification, thereby reducing the accumulation of nitrate and lowering water demand in RAS. Although fibers were partially degraded in the reactors, they still remain a major limitation for carbon bioavailability in fecal waste. It was not possible to develop a treatment to increase the bioavailability of fibers for denitrification. To ensure an efficient use of carbon which is already bioavailable, oxidative losses in denitrfication reactors need to be minimized. As feed is the principal source of waste in RAS, a more selective choice of plant ingredient can improve the recovery and degradability of fecal waste.

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Summary

Due to the depletion of global fish stocks and increasing fish meal prices, fish meal is being increasingly substituted by unpurified plant ingredients as an alternative source of protein in fish feeds. Fibers originating from these plant ingredients are mostly indigestible and increase the production of fecal waste in aquaculture. Recirculating aquaculture systems (RAS) rely on an efficient removal of fecal waste from the systems to maintain water quality and biofilter performance (**Chapter 1**). However fibers originating from the feed can affect the recovery characteristics of fecal waste by changing particle size, density and pellet stability. Thus, the substitution of fish meal with unpurified plant ingredients will not only increase waste production, but could also affect the performance of RAS. To counteract an increased solid waste production in RAS, fecal waste can be used as a carbon source in denitrification. However, it is not clear whether the low degradability of fibers will hamper the utilization of fecal carbon in denitrification . Therefore, this thesis investigated the effect of fibers in fish feeds on fecal waste production, recovery and degradability in RAS. Furthermore, we investigated if ultrasound- or enzyme treatment can be used to overcome a limited carbon bioavailability in fecal waste.

In **Chapter 2**, two diets with a contrast in fiber content were used to study the effect of fibers on fecal waste production, recovery and degradability. The experiment was performed in six small-scale RAS (V= 460 L) stocked with rainbow trout (*Oncorhynchus mykiss*). Using digestibility data, the high fiber diet (HNSP) produced approximately 50% more fecal organic carbon (chemical oxygen demand, COD) than the low fiber diet (LNSP). Furthermore, the recovery of HNSP feces (79%) was significantly higher than the recovery of LNSP feces (56%). This resulted in a significantly lower effective load of degradable organic matter (BOD_5) on the biofilters in HNSP systems (0.6 g BOD_5/g N) when compared to LNSP systems (1.1 g BOD_5/g N). Both feeds produced an equal amount of biodegradable COD per kg of feed (107 g BOD_{10}/kg feed DM). Only the HNSP diet produced sufficient COD to theoretically allow for full nitrate removal in denitrification (7.2 g COD/g N for HNSP, vs. 3.4 g COD/g N for LNSP). However, the low degradability of HNSP feces suggests that fibers would become a limiting factor for denitrification on internal carbon sources (3.1 g BOD_{10}/g N).

In **Chapter 3**, the HNSP and LNSP diet were used to study the effects of fibers on particle size distribution in fecal waste. Furthermore, we tested whether ultrasound treatment can decrease particle size and thus increase the short-term degradability in fecal waste. Fecal waste was collected in flow-through tanks stocked with rainbow trout. Particle size distribution was determined by wet sieving, using different screen sizes between 1000

and 32 µm. COD short-term bioavailability was determined in oxygen uptake rate (OUR) tests. The collected feces were treated with high intensity, low-frequency ultrasound to determine whether this can reduce particle size in fecal waste and increase COD bioavailability. The median particle size was significantly higher in HNSP feces (101 µm) than in LNSP feces (24 µm). Subsequently, approximately 50% of the dry matter from fully disintegrated HNSP could be retained on a microscreen of 36 µm, whereas it was only ~10% for LNSP feces. This indicates that the fibers in HNSP feces could improve fecal waste recovery for drum filtration in RAS. However, both diets produced the same amount of particles, which could pass a drum filter screen and could thus potentially accumulate in RAS (~50 g/kg feed DM). Ultrasound had only a minor effect on particle size and biodegradability, which suggests that it is not a suitable pre-treatment method to increase carbon bioavailability in fecal waste for denitrification. This in turn indicates, that the particles forming the fecal pellet are extremely resilient to mechanical stress.
The role of fibers as a carbon source for denitrification was investigated in **Chapter 4**. This was studied in four small-scale RAS stocked with rainbow trout, which were fed with the HNSP diet. Two RAS were equipped with an upflow sludge blanket denitrification reactor and two RAS served as control systems without denitrification. The production of metabolic waste per kg feed DM was determined in the control systems, and compared with the total COD and N production in the system with denitrification. The reactors were able to use 44% of the fecal COD for denitrification, which subsequently decreased N production in RAS with denitrification by 50%. Although cellulose and hemicellulose degradability was 50% and 60% respectively, fibers were still a major limitation for carbon bioavailability in denitrification on internal carbon sources. Approximately 4.4 g of biodegradable COD were necessary to remove 1 g of nitrate-N. This indicates that only half of the bioavailable carbon present in fecal waste was used for denitrification, the rest was presumably respired with dissolved oxygen entering the reactor with the sludge flow.
In **Chapter 5**, an enzyme mixture (Viscozyme® L) was used to test whether the fibers in HNSP feces can be hydrolyzed to increase carbon bioavailability for denitrification. In the first period of the experiment, the optimal hydrolysis conditions for the enzyme were determined using dried fecal waste collected in flow-through tanks. The results showed that the enzyme was able to hydrolyze 22% of the fecal carbohydrate fraction under optimal conditions, despite the observed lack of cellulolytic activity. In the second period of the experiment, the effect of enzyme treatment on the degradability of fibers in fecal waste was investigated. Two RAS with upflow sludge blanket denitrification reactors were operated at the same time and under similar conditions as the experiment in Chapter 4. The fecal waste, which was produced in these experimental systems, was collected and treated with the enzyme for 24 hours. Then, the treated fecal waste was fed batch-wise into the denitrification reactors. Although the enzyme was able to hydrolyze a part the fibers present in fecal waste, this did not improve the degradability of fecal waste in the denitrification reactors. This indicates that the degradation of fibers is not a linear process and that fiber degradation is probably limited by structural properties as lignification or

cellulase accessibility. To effectively increase carbon bioavailability for denitrification, a suitable enzyme should be able to hydrolyze more than 50% of the cellulose and 60% of the hemicellulose present in fecal waste. The residual solids remaining in the reactor after digestion could be used to screen for enzymes, which are able to degrade the recalcitrant fiber fraction in fecal waste.

The results obtained in this thesis can help to understand the future challenges of fish meal substitution by unpurified plant ingredients (**Chapter 6**). Fibers decrease the dry matter digestibility of the feeds and subsequently increase solid waste production in RAS. However, fibers originating from unpurified plant ingredients can also improve fecal waste removal in RAS. This in turn has a positive effect on system performance and water quality, as the organic load on the biofilters is reduced. To abate the problem of an increased solid waste production in RAS, fecal waste can be used as a carbon source for denitrification. However, the utilization of fecal waste as a carbon source is hampered by the low degradability of fibers. Since no suitable conditioning method was identified to increase the degradability of fibers, the problem of an increased production of solid waste still remains an issue for denitrification on internal carbon sources and other waste treatments alike. However, improving the design of denitrification reactors can significantly improve the efficient use of degradable carbon for denitrification in RAS. A more selective choice of feed ingredients may increase fecal waste recovery and the degradability of fibers, which can ultimately improve system performance and reduce solid waste emissions from RAS.

Samenvatting

Als gevolg van de uitputting van mondiale visbestanden door de visserij en de stijgende prijzen voor vismeel, wordt vismeel in voeder voor vissen steeds vaker vervangen door ongezuiverde plantaardige ingrediënten als een alternatieve bron van eiwitten. Vezels die afkomstig zijn van deze plantaardige ingrediënten zijn meestal onverteerbaar voor vissen en verhogen daardoor de productie van fecale afvalstoffen in de aquacultuur. Recirculatie aquacultuur systemen (RAS) zijn voor het in stand houden van de waterkwaliteit en het optimaal functioneren van het biofilter gebaat bij een hoge verwijderingsefficiëntie van deze fecale afvalstoffen (hoofdstuk 1). Echter, vezels afkomstig uit het voer kunnen de verwijderingskarakteristieken van de geproduceerde fecale afvalstoffen beïnvloeden door de deeltjesgrootte, de dichtheid en fecale pellet stabiliteit te veranderen. Dus, de vervanging van vismeel door ongezuiverde plantaardige ingrediënten zorgt niet alleen voor een toename in de afvalproductie, maar kan ook van invloed zijn op de prestaties van een recirculatie systeem met betrekking tot het in stand houden van de waterkwaliteit. Om de toename van de vaste afvalproductie in RAS tegen te gaan, kan het fecale afval worden gebruikt als een koolstofbron in het denitrificatieproces. Het is echter onduidelijk of de lage afbreekbaarheid van vezels de beschikbaarheid van fecale koolstof in het denitrificatie process zal beperken. Dit PhD-thesis project onderzocht daarom het effect van vezels in visvoeder op de fecale afvalproductie, de verwijderingsefficientie van fecale afvalstoffen en de afbreekbaarheid ervan in recirculatie aquacultuur systemen. Bovendien werd onderzocht of ultrasound- of enzym behandeling kunnen worden gebruikt om beperkte biologische beschikbaarheid van koolstof in het fecale afval te verminderen.
In **hoofdstuk 2** werden twee diëten met een contrast in vezelgehalte gebruikt voor het bestuderen van het effect van vezels op fecale afvalproductie, de verwijderingsefficientie en biologische afbreekbaarheid daarvan. Het experiment werd uitgevoerd in zes kleinschalige RAS (V= 460 L) bezet met regenboogforel (*Oncorhynchus mykiss*). Met behulp van de gemeten verteerbaarheidwerd uitgerekend dat vissen gevoerd met het vezelrijke dieet (HNSP) ongeveer 50% meer fecale organische koolstof (chemisch zuurstof verbruik, CZV) produceerden dan vissen gevoerd met het lage vezel dieet (LNSP). Bovendien was de verwijderingsefficiëntie voor de fecale afvalproductie op het HNSP dieet aanzienlijk hoger (79%) dan die voor het LNSP dieet (56%). Dit resulteerde voor de HNSP systemen in een aanmerkelijk lagere belasting van de biofilters met afbreekbaar organisch materiaal (BZV_5; 0,6 g BZV_5/g N) in HNSP in vergelijking met de LNSP systemen (1,1 g BZV_5/g N). Op beide voeders werd door forel eenzelfde hoeveelheid biologisch afbreekbare CZV per kg voer geproduceerd (107 g BZV_{10}/kg feed DM). Alleen op het HNSP dieet

werd voldoende CZV geproduceerd om theoretisch volledige nitraatverwijdering door denitrificatie mogelijk te maken (7,2 g CZV/g N voor HNSP, vs. 3,4 g CZV/g N voor LNSP). Echter, de lage afbreekbaarheid van het fecale afval geproduceerd op het HNSP dieet duidt erop dat vezels een beperkende factor voor koolstof beschikbaarheid in interne afval bronnen (3,1 g BZV_{10}/g N) zouden kunnen worden.
In **hoofdstuk 3** werden de HNSP en LNSP dieten gebruikt voor het bestuderen van de effecten van vezels op deeltjes grootte verdeling in de fecale afvalproductie. Bovendien testen we of ultrasound behandeling de fecale deeltjes kan verkleinen om zo op de korte termijn de afbreekbaarheid van fecale afvalproductie te verhogen. De fecale afvalproductie per dieet werd verzameld in doorstroom tanks bezet met regenboogforel. Deeltjes grootte verdeling in het fecale afval werd bepaald door nat zeven met behulp van verschillende zeefgroottes tussen 1000 µm en 32 µm. Het aandeel direct biologisch afbreekbare CZV werd bepaald door middel van zuurstof opname snelheidstesten ('OUR-tests'). De verzamelde feces werden behandeld met hoge intensiteit, lage frequentie ultrasound om te bepalen of dit de grootte van de deeltjes in het fecale afval verkleinde en de hoeveelheid direct biologisch beschikbare CZV in de feces kan verhogen. De gemiddelde deeltjesgrootte was significant hoger in de feces geproduceerd op het HNSP dieet (101 µm) dan op het LNSP dieet (24 µm). Vervolgens kon na nat zeven 50% van de droge stof in de volledig gedesintegreerde HNSP feces worden verzameld op een microscreen van 36 µm, terwijl dit voor LNSP feces slechts 10% was. Dit geeft aan dat de vezels in de HNSP feces de verwijderingsefficientie van feces in drumfilters in recirculatie systemen zouden kunnen verbeteren. Echter, beide diëten produceerden dezelfde hoeveelheid deeltjes die een drumfilter kunnen passeren en dus potentieel kunnen ophopen in een recirculatie systeem ($\tilde{5}$0 g/kg drogestof in het voer). Ultrasound behandeling had slechts een klein effect op de deeltjesgrootte en biologische afbreekbaarheid van de feces. Dit geeft aan dat het niet een geschikte voorbehandelingsmethode is om de biologische beschikbaarheid van koolstof in feces ten behoeve van denitrificatie te verhogen. Dit geeft tevens aan dat de deeltjes die samen de fecale pellet vormen uiterst bestendig zijn tegen blootstelling aan mechanische stress.
De rol van vezels als een koolstofbron voor denitrificatie werd onderzocht in **hoofdstuk 4**. Dit werd bestudeerd in vier kleinschalige recirculatie systemen bezet met regenboog forel die gevoerd werden met het HNSP dieet. Twee RAS waren uitgerust met een opstroom slibbed denitrificatie reactor en twee RAS dienden als controlesystemen zonder denitrificatie. De productie aan metabolische afvalstoffen per kg voer drogestof werd bepaald in de controlesystemen en vergeleken met de totale productie aan COD en stikstof (N) in de systemen met een denitrificatie reactor. De denitrificatie reactoren waren in staat om 44% van de fecale CZV te gebruiken voor denitrificatie waardoor de N productie in recirculatie systemen met denitrificatie met 50% daalde. Hoewel de afbreekbaarheid van cellulose en hemicellulose respectievelijk 50% en 60% was, waren vezels nog steeds een belangrijke beperking voor biologische beschikbaarheid van koolstof voor denitrificatie op interne koolstof bronnen. Ongeveer 4,4 g van biologisch afbreekbaar CZV was nodig

om 1 g nitraat stikstof te verwijderen. Dit geeft aan dat slechts de helft van de biologisch beschikbare koolstof aanwezig in fecale afvalstoffen werd gebruikt voor denitrificatie, de rest werd vermoedelijk geoxideerd met zuurstof in de ingaande slibstroom van de denirificatie reactor.
In **hoofdstuk 5** werd een mengsel van enzymen (Viscozyme® L) gebruikt om te testen of de vezels in de fecale afvalstoffen geproduceerd op het HNSP dieet kunnen worden gehydrolyseerd om zo de biologische beschikbaarheid van koolstof ten behoeve van het denitrificatie process te verhogen. In de eerste periode van het experiment werden de optimale hydrolyse condities voor het enzym bepaald met behulp van gedroogde fecale afvalstoffen van het HNSP dieet, verzameld in doorstroom tanks bezet met forel. Ondanks het waargenomen gebrek aan cellulolytische activiteit lieten de resultaten zien dat het enzym onder optimale hydrolyse condities in staat was om 22% van de fecale koolhydraten te hydrolyseren. Dit ondanks het waargenomen gebrek aan cellulolytische activiteit. In de tweede periode van het experiment werd het effect van de enzymbehandeling op de afbreekbaarheid van vezels in fecale afvalstoffen onderzocht. Twee RAS met upflow slibbed denitrificatie reactoren werden tezelfdertijd en onder dezelfde omstandigheden gebruikt als de denitrificatie reactoren in het experiment in hoofdstuk 4. De dagelijks in deze experimentele recirculatie systemen geproduceerde fecale afvalstoffen werden verzameld en met het enzym behandeld gedurende 24 uur. Vervolgens werd het enzym behandelde fecale afval batchgewijs in de denitrificatie reactoren gevoerd. Hoewel het enzym een deel van de aanwezige vezels in het fecale afval kon hydrolyseren, gaf dit geen verbetering van de afbreekbaarheid van het fecale afval in de denitrificatie reactoren wanneer vergeleken met denitrificatie reactoren waarin geen enzym behandeling was toegepast. Dit geeft aan dat de afbraak van vezels niet een lineair proces is en dat de afbraak van de vezels waarschijnlijk wordt beperkt door structuureigenschappen zoals lignificatie of de toegangkelijkheid van cellulase. Om effectief de biologische beschikbaarheid van koolstof voor denitrificatie te verhogen, moet een geschikt enzym meer dan 50% van de cellulose en 60% van de hemicellulose aanwezig in de geproduceerde fecale afvalstoffen kunnen hydrolyseren. Voor het screenen van enzymen die in staat zijn deze recalcitrante vezelfractie af te breken kunnen dee resterende vaste delen van de fecale afvalstoffen die na vertering in de denitrificatie overblijven worden gebruikt.
De verkregen resultaten in deze PhD-thesis kunnen helpen bij toekomstige uitdagingen om vismeel te vervangen door ongezuiverde plantaardige ingrediënten (**hoofdstuk 6**). Vezels verminderen de verteerbaarheid van de droge stof in voeders en verhogen vervolgens de vaste afvalstoffen productie in recirculatie systemen. Echter, vezels van oorsprong afkomstig uit ongezuiverde planten ingrediënten kunnen ook de fecale afvalstoffen verwijdering uit RAS verbeteren. Dit heeft op zijn beurt een positief effect op de prestaties van en de waterkwaliteit in een recirculatie systeem, aangezien de organische stof belasting op de geïnstalleerde biofilters in RAS wordt verminderd. Om het probleem van een verhoogde vaste afvalstoffen productie in RAS tegen te gaan, kunnen fecale afvalstoffen worden gebruikt als een koolstofbron voor denitrificatie. Echter, het gebruik

van fecale afvalstoffen als koolstofbron voor denitrificatie wordt belemmerd door de lage afbreekbaarheid van vezels. Omdat geen geschikte conditionering methode werd geïdentificeerd om de afbreekbaarheid van vezels te verhogen, blijft een verhoogde productie van vast afval nog een probleem voor denitrificatie en andere afvalbehandelingen op de interne koolstofbron. Echter, verbetering van het ontwerp van denitrificatie reactoren kan het efficiënt gebruik van afbreekbaar koolstof voor het denitrificatie proces in RAS aanzienlijk verbeteren.
Een meer selectieve keuze van voederbestanddelen kan de verwijderingsefficientie van de vaste afvalstoffen en de afbreekbaarheid van vezels verhogen, welke uiteindelijk de recirculatiesysteem prestaties kunnen verbeteren en de uitstoot van vaste afvalstoffen uit RAS kunnen verminderen.

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Disclaimer

If you don't find your name on the list, it does not mean that your contribution was not appreciated. This in fact means, that if you have a justified claim on being acknowledged in this thesis, we can arrange a meeting including a free beverage of your choice!

About the author

Andre Meriac was born in Brackenheim, Germany, on January 19th, 1983. His scientific career began in 2003, when he started his study of biology at the University of Konstanz, Germany. The focus of the basic studies was on molecular biology, microbiology and biochemistry. During his main studies, he focused on ecotoxicology, microbiology, limnology and fish ecology. Driven by his enthusiasm for the aquatic environment, he also worked during this time as a student research assistant at the Limnological Institute. There, he was assisting with field work, sample preparation and data analyses at the fish ecology group under Prof. Dr. Reiner Eckmann. In 2008, he finished his studies with a thesis on the role of MHC-alleles on shoaling decisions in juvenile perch (*Perca fluviatilis*), and subsequently received his degree as Diplom-Biologe (MSc equivalent).

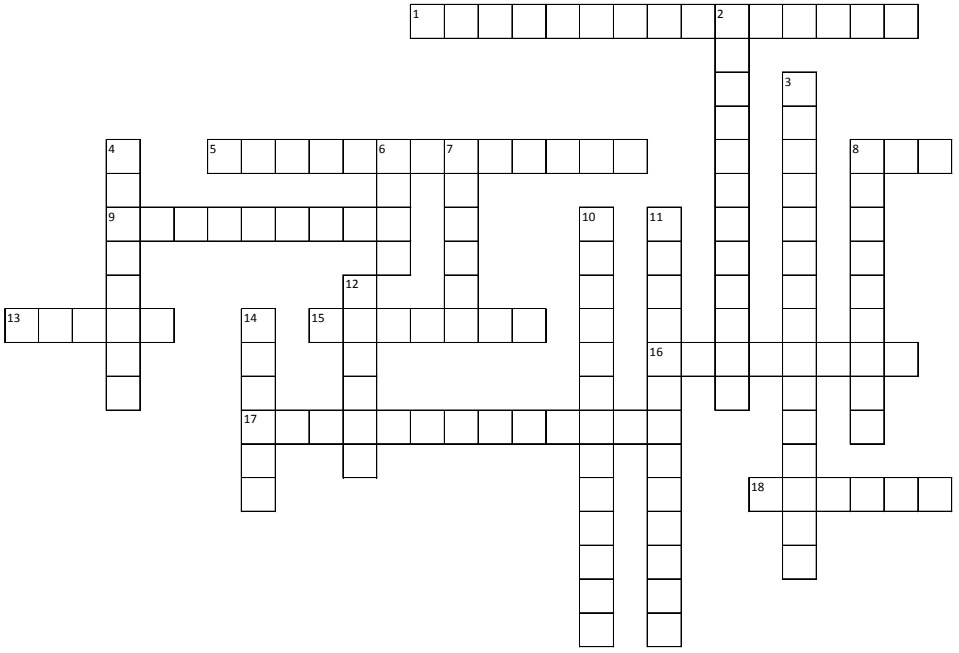
After his graduation, he continued working at the Limnological Institute and was responsible for the analysis of molecular markers for phylogenetic research on Chironomid communities. In search for more applied research, he started a self-funded internship (Leonardo da Vinci scholarship) at the Institute for Marine Resources & Ecosystem Studies (IMARES) in Yerseke in the beginning of 2009. He worked on the development of a setup for long-term transport of sturgeon, and also participated in other contract research activities. At the end of this year, he started his PhD research at IMARES in cooperation with the Aquaculture & Fisheries group at Wageningen University. The practical goal of this project was the reduction of waste emissions from recirculating aquaculture systems. During this time, he successfully presented his research at international conferences and published in peer-reviewed journals. At the World Aquaculture Society meeting in 2012 (Prague, Czech Republic), his presentation on the effect of plant-based diets on COD/N-ratios for denitrification was awarded the 2nd place in the student spotlight competition. After finishing his PhD, he will be following his passion for applied, interdisciplinary research.

Contact: research.meriac@gmail.com

WIAS training & supervision plan

Education and training	ECTS
The basic package	**3**
WIAS introduction course	
Ethics & philosophy in animal science	
Scientific exposure	**10**
IMARES PhD Day 2011 (oral presentation)	
Aquaculture Europe 2012, Prague, Czech Republic (oral presentation)	
Aquaculture Europe 2012, Prague, Czech Republic (student spotlight competition)	
International Conference on Recirculating Aquaculture 2012, Roanoke, USA (ppt slides, presented by Ep H. Eding)	
Aquaculture Europe 2013,Trondheim, Norway (oral presentation)	
2nd Workshop on RAS 2013, Aalborg, Denmark (oral presentation)	
In-depth studies	**7**
RAS workshop at EAS 2012, Prague, Czech Republic	
AquaExcel RAS workshop 2013, Wageningen, The Netherlands	
Masterclass Biobased Innovation	
Advanced statistics: Design of Experiments	
Statistics for the Life Sciences	
Statutory Courses	**3**
Use of Laboratory Animals	
Professional Skills Support Courses	**4**
Scientific writing	
Presentation skills	
Project & time management	
Didactic & research skills training	**16**
Preparing own research proposal	
Supervision of 5 MSc major students	
Education & training total	**43**

Quiz page



Across:

1. Microbial conversion of nitrate to N_2

5. Ammonia converting process in RAS biofilters

8. Indirect measure for organic carbon

9. Enzyme to facilitate cellulose degradation

13. Undigested feed

15. Feed additives which can increase feces recovery

16. Common external carbon source for denitrification

17. Re-use of water

18. _____ limit the bioavailability of carbon in fecal waste

Down:

2. Type of macro-molecule, consisting of carbon, hydrogen and oxygen

3. Determines how much of a carbon source can be utilized by bacteria

4. Determines organic load on biofilters in RAS

6. Main source of waste in RAS

7. Catalyst for PhD theses

8. Polysaccharide, main constituent of cell walls in plants

10. Determines fecal waste production

11. Solids collection by gravity

12. Cell wall constituent of especially low degradability

14. Easily degradable carbohydrate in feed and feces

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The salmon used for the cover design and for illustrations in this thesis is based on the artwork of Timothy Knepp, which was published as public domain by the U.S. Fish and Wildlife Service.

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